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The absence of PNPase activity in *Enterococcus faecalis* results in alterations of the bacterial cell-wall but induces high proteolytic and adhesion activities.

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2 **Rabia Ladjouzi*, Matthieu Duban, Anca Lucau-Danila, Djamel Drider**

3

4 UMR Transfrontalière BioEcoAgro N° 1158, Univ. Lille, INRAE, Univ. Liège, UPJV,
5 YNCREA, Univ. Artois, Univ. Littoral Côte d'Opale, ICV – Institut Charles Viollette, F-59000
6 Lille, France

7

8 *** Corresponding author:**

9 Rabia LADJOUZI. Université de Lille, cité scientifique/Avenue Paul Langevin, Polytech-Lille,
10 Bureau C310. 59655 Villeneuve d'Ascq, France. Email. rabia.ladjouzi@univ-lille.fr

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12

13 **Abstract**

14 Enterococci are lactic acid bacteria (LAB) used as starters and probiotics, delineating their
15 positive attributes. Nevertheless, enterococci can be culprit for thousands of infectious diseases,
16 including urinary tract infections, bacteremia and endocarditis. Here, we aim to determine the
17 impact of polynucleotide phosphorylase (PNPase) in the biology of *Enterococcus faecalis* 14; a
18 human isolate from meconium. Thus, a mutant strain deficient in PNPase synthesis, named
19 $\Delta pnpA$ mutant, was genetically obtained. After that, a transcriptomic study revealed a set of 244
20 genes differentially expressed in the $\Delta pnpA$ mutant compared with the wild-type strain, when
21 exploiting RNAs extracted from these strains after 3 and 6 hours of growth. Differentially
22 expressed genes include those involved in cell wall synthesis, adhesion, biofilm formation,
23 bacterial competence and conjugation, stress response, transport, DNA repair and many other
24 functions related to the primary and secondary metabolism of the bacteria. Moreover, the $\Delta pnpA$
25 mutant showed an altered cell envelope ultrastructure compared with the WT strain, and is also
26 distinguished by a strong adhesion capacity on eukaryotic cell as well as a high proteolytic
27 activity. This study, which combines genetics, physiology and transcriptomics enabled us to show
28 further biological functions that could be directly or indirectly controlled by the PNPase in *E.*
29 *faecalis* 14.

30 **Keywords:** polynucleotide phosphorylase (PNPase), transcriptomic analysis, cell envelope
31 alterations, proteolytic activity, cell adhesion

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35 1. Introduction

36 Polynucleotide phosphorylase (PNPase) is conserved in the course of evolution and
37 therefore is found in different lineages of life as in Bacteria, Archaea, Eukarya, organelles,
38 animals and plants (Leszczyniecka et al., 2004). In terms of its enzymatic activity, two types of
39 reactions associated with PNPase were demonstrated. The first consists in the processive
40 phosphorolytic degradation of mRNA from the 3'-end. Thus, PNPase catalyzes stepwise
41 phosphorolysis of the 3'-terminal phosphodiester of RNA chains leading to nucleoside
42 diphosphate products (Arraiano et al., 2010). The second consists on a polymerase activity
43 catalyzing reaction using NDPs as substrates to add NMPs to the 3'-end of the RNA chain
44 (Mohanty and Kushner, 2000). The activity of PNPase can be hampered by well-folded RNAs,
45 referred to as double-helical structured RNAs (Spickler and Mackie, 2000). To get over this
46 hurdle, PNPase has a potential to bind to other proteins, forming thereof complex machineries
47 capable of coming through these well-structured RNAs (Arraiano et al., 2010).

48 In Gram-positive bacteria such as *B. subtilis*, it was established that the formation of the
49 degradosome complex is initiated by RNase Y that binds to exoribonucleases PNPase, to RNase
50 J1 and J2, and then to the other proteins of the complex (Cascante-Esteva et al., 2016; Lehnik-
51 Habrink et al., 2012; Salvo et al., 2016). In *B. subtilis*, the efficient exonucleolytic mRNA decay
52 was found to be dependent on the conjunction of PNPase and helicase CshA (Cardenas et al.,
53 2011), advocating the complexity of the machineries of mRNA degradation. Of note in
54 *Staphylococcus aureus*, PNPase was also shown to interact with RNase Y to degrade transcripts
55 (Numata et al., 2014), whilst in *Streptococcus pyogenes*, PNPase was found to be the main 3'-to-

56 5' *in-vivo* exoRNase degrading fragments generated by the RNase Y endoRNases during mRNA
57 decay (Broglia et al., 2020; Lécivain et al., 2018).

58 Bacterial species lacking PNPase synthesis are affected in an arc of functions and become
59 unable to execute correctly their genetic programs. In addition to proceed with the possessing of
60 mRNAs in Gram-positive and Gram-negative bacteria, PNPase was shown to be directly or
61 indirectly involved in bacterial responses to cold shock (Zangrossi et al., 2000), cell-competence
62 (Luttinger et al., 1996), biofilm formation (Carzaniga et al., 2012), oxidative and other
63 environmental stress (Wu et al., 2009; Wurtmann and Wolin, 2009), and virulence (Rosenzweig
64 and Chopra, 2013; Sinha et al., 2021). Interestingly, a potential link could exist between PNPase
65 and DNA metabolism as formerly reported, suggesting that PNPase can reversibly catalyze
66 phosphorolysis of ssDNA, and having a role in the DNA repair, mutagenesis and recombination
67 (Becket et al., 2012; Cardenas et al., 2011, 2009; Rath et al., 2012).

68 In enterococci, only few studies have been conducted on enzymes involved in RNA
69 metabolism. Recently, the role of RNases (J1, J2, III and Y) and three DEAD-box helicases
70 (CshA, B and C) on physiology and virulence of *E. faecalis* was reported (Gao et al., 2017; Salze
71 et al., 2020). In the present work, we focus on the PNPase from *E. faecalis* 14; a strain formerly
72 isolated from meconium (Al Atya et al., 2015). In a first step, we performed a transcriptomic
73 study to identify the genes or network of genes that could be under the direct or indirect control
74 of PNPase, which is known to be a pleiotropic enzyme. For this purpose, we constructed a mutant
75 deficient in PNPase synthesis ($\Delta pnpA$) by using the double homologous recombination method.
76 RNAs extracted from the $\Delta pnpA$ mutant and the WT strains, after 3 and 6 hours of growth were
77 therefore used for this transcriptomic study.

78 This study clearly shows that the absence of PNPase affects the ultrastructure of the
79 bacterium by altering its cell-envelope, as well as by reducing the size of the cells and colonies.
80 However, these alterations were not detrimental as we recorded better scores of adhesions on
81 Caco-2 eukaryotic cells as well as higher proteolytic activity in the $\Delta pnpA$ mutant strain than the
82 WT strain. An attempt to link these structural changes and enhanced biological activities through
83 the interpretation of transcriptomic data has been proposed.

84 **2. Materials and methods**

85 **2.1. Bacterial strains and growth conditions**

86 Tables 1 and 2 list all bacterial strains, plasmids and primers used in this study. *E. faecalis*
87 14 cultures were grown at 37 °C in GM17 (M17 medium containing 0.5% (w/v) of glucose).
88 When needed, chloramphenicol (Cm) or erythromycin (Er) antibiotics were added to the media at
89 final concentration of 15 $\mu\text{g ml}^{-1}$ or 150 $\mu\text{g/mL}$ respectively. The agar plates were used for
90 determining the cfu counts. The kinetic growth was performed using the spectrophotometer
91 (Aquoalabo, France) set at an optical density of 600 nm (OD_{600}). *E. coli* strains were grown at 37
92 °C in Luria-Bertani (LB) broth under aerobic condition with shaking at 160 rpm or on LB agar.
93 When appropriate, ampicillin (100 $\mu\text{g/mL}$), erythromycin (150 $\mu\text{g/mL}$) or chloramphenicol (10
94 $\mu\text{g/mL}$) was added.

95 96 **2.2. General molecular methods**

97 Molecular cloning and standard techniques and were routinely performed as described
98 by(Sambrook and Russell, 2001). All enzymes were used in accordance with the manufacturer's
99 instructions and obtained from ThermoFisher Scientific (Courtaboeuf, France). NucleoSpin® kits
100 (Macherey-Nagel, Düren, Germany) was used to purify PCR products and plasmids. All genetic

101 constructions and the resulting mutants were sequenced (Eurofins, Ebersberg, Germany).
102 SnapGenes tool (GSL Biotech LLC, CA) was used to analyze DNA sequences. Transformation
103 of *E. coli* and *E. faecalis* strains were performed respectively by heat shock and by
104 electroporation using the Gene Pulser Apparatus (Bio-Rad, Hercules, CA, USA).

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2.3. Construction of the $\Delta pnpA$ mutant strain

107 The $\Delta pnpA$ mutant strain was constructed by double cross-over using a strategy based on
108 the conditional replication of the pLT06 plasmid (Thurlow et al., 2009), as previously described
109 (Ladjouzi et al., 2020a). Briefly, regions of 953 bp located upstream and 966 bp downstream of
110 the *pnpA* gene were amplified by PCR using the primer pairs *pnpA*-1F /*pnpA*-2R and *pnpA*-
111 3F/*pnpA*-4R respectively (Table 2). The *pnpA*-3F and *pnpA*-2R primers are complementary. The
112 DNA fragment harboring a deletion of 1,148 pb in the N-terminal region of *pnpA* gene was
113 generated with a second PCR using the primers *pnpA*-1F / *pnpA*-4R and the mix of the two
114 previous amplified fragments as DNA template. The resulting DNA fragment of 1.919 bp was
115 cloned into pGEM-t Easy vector (Promega, Madison, WI, USA). The white colonies of *E. coli*
116 XL-1 Blue obtained on LB-X-Gal (40 μ g/mL - IPTG (40 μ g/mL) containing the recombinant
117 plasmid pGEM-t Easy $\Delta pnpA$ were selected. The fragment containing mutated *pnpA* gene was
118 extracted from pGEM-t Easy $\Delta pnpA$ using *Pst*I and *Nco*I restriction enzymes and cloned into the
119 pLT06 plasmid to generate pLT06 $\Delta pnpA$ using the *E. coli* JM109 competent cells for the
120 transformation. The pLT06 $\Delta pnpA$ plasmid was used to transform *E. faecalis* 14 WT and the
121 crossing-over events were induced as previously described (Ladjouzi et al., 2020a). The deletion
122 of the *pnpA* gene was screened by PCR using *pnpA*5F/*pnpA*6R or *pnpA*7F/*pnpA*8R and
123 confirmed by sequencing.

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2.4. Complementation of *E. faecalis* $\Delta pnpA$ mutant strain

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A DNA fragment of 2.32 kb containing the entire *pnpA* gene including the promoter region was amplified by PCR using *pnpA*-CompF and *pnpA*-CompR primers and cloned into pMSP3535 (Tables 1 and 2). The recombinant plasmid pMSP3535:*pnpA* was used to transform *E. faecalis* 14 $\Delta pnpA$ mutant after transformation in *E. coli* XL-1 blue and selection of the positive colonies on LB, Er (150 $\mu\text{g ml}^{-1}$). After 48 h at 37°C, colonies carrying the recombinant plasmid pMSP3535:*pnpA* were selected on GM17 Er (150 $\mu\text{g/mL}$) then screened by PCR and sequenced.

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2.5. Transmission electron microscopy

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For TEM analysis, pellets of WT and its $\Delta pnpA$ mutant were obtained by centrifugation (8,000 \times g, 10 min, 4 °C) of cultures grown on GM17 at 37 °C for 6 h. Pellets were fixed and TEM images were obtained using the same method previously described (Ladjouzi et al., 2020b). An acceleration voltage of 200 KV of the microscope JEOL JEM 2100FX TEM instrument (Jeol, Tokyo, Japan) was used.

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2.6. Microarrays analysis and RNA isolation

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The microarray analysis was performed using three distinct cultures of *E. faecalis* 14 $\Delta pnpA$ which were compared with *E. faecalis* 14, after 3 h and 6 h of growth in GM17 medium. Cells were harvested by centrifugation (10,000 \times g during 10 min at 4°C) and total RNA was extracted using the NucleoSpinTM RNA Plus columns (Macherey-Nagel, Hoerd, France). The quantity and quality of RNA samples were determined by capillary electrophoresis, using an

147 Agilent 2100 Bioanalyzer (Agilent Technologies, France) and a minimal RNA integrity number
148 (RIN) of 8 was required for all samples.

149 A custom *E. faecalis* 14 oligo-based DNA microarray (8 × 15 K) Agilent G2509F was
150 used to study the gene expression according to the method previously developed in our previous
151 work (Ladjouzi et al., 2020b). Differentially expressed genes (DEGs) were selected when FC
152 threshold > 2.0 or < 0.5 and presented in log₂ FC. For the functional annotation of DEGs and
153 relevant biological pathways of selected genes NCBI GenBank, UniProt, AmiGO 2 Gene
154 Ontology and KEGG pathway analysis were used. The microarray data from this study have been
155 submitted to the NCBI GEO with the accession number GSE180397.

156

157 **2.7. Adhesion to Human Intestinal Cells Caco-2**

158 For the adhesion assay, the human colorectal adenocarcinoma Caco-2 cells were used
159 (Pinto et al., 1983) according to the previously described protocol (Bendali et al., 2011). Briefly,
160 cells were grown at 37 °C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM)
161 Gibco® (Thermo Fisher Scientific), supplemented with 4.5 g/L glucose, 2 mM L-glutamine, 1%
162 (v/v) non-essential amino acids, 100 U/mL streptomycin, 100 U/mL penicillin, and 10% (v/v)
163 heat-inactivated fetal bovine serum. The reagents used were from PAN-Biotech GmbH
164 (Aidenbach, Germany). To measure the adhesion ability of the bacteria, a culture of 7 days of
165 Caco-2 cells was performed in 24-well tissue culture plates using an inoculum of 5.10⁴ cells per
166 well. For the bacteria, 10 mL of overnight cultures of the *E. faecalis* WT and $\Delta pnpA$ mutant
167 strains grown on GM17 (~ 6.10⁹ cfu/mL) were harvested by centrifugation (6,000 × g, 4 °C, 10
168 min), washed twice with PBS, resuspended in 10 mL of non-supplemented DMEM, and applied
169 to confluent Caco-2 cell monolayers (~ 5.10⁵ per well). Volumes of 200 μL and 300 μL

170 containing 6.10^9 cfu/mL were used. After 2 h of incubation at 37 °C, Caco-2 cell monolayers
171 were washed twice with 500 μ L of 10 mM PBS (pH 7.2) to remove non-adherent bacteria. Then,
172 200 μ L trypsin/EDTA Gibco® (Thermo Fisher Scientific) were used to lyse cells for 15 min at
173 room temperature. Finally, the lysates were diluted and the number of adherent bacteria was
174 determined on GM17 agar. Three independent experiments were performed and three replicates
175 by condition were done for each experiment. The percentage of adhesion was determined by the
176 ratio of adherent bacteria/total number of bacteria (control) multiplied by 100.

177

178 **2.8. CCK-8 cytotoxicity assay**

179 The Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technology, Japan) was
180 used to assess the cytotoxicity of WT and its $\Delta pnpA$ mutant strains against Caco-2 cells, as
181 previously described (Ladjouzi et al., 2020b). A culture of 7 days of Caco-2 cells at a density of 6
182 $\times 10^4$ cells/well was carried out in 96-well cell culture plates using the same conditions as cited
183 above. Overnight cultures grown in GM17 were centrifuged and resuspended in DMEM without
184 antibiotic using the same initial volume. Then, bacteria were added to Caco-2 cell monolayers at
185 a ratio of MOI (Multiplicity of infection) 1:10 (Caco-2/WT or Caco-2/ $\Delta pnpA$ mutant). The non-
186 infected Caco-2 cells were used as control. After 24 h of incubation, cells were washed twice
187 with PBS and incubated for 2 h at 37 °C in 150 μ L of DMEM and 5% of CCK-8 reagent. The
188 relative viability (%) of Caco-2 cells was determined by absorbance values at 450 nm using a
189 microplate reader (Xenius SAFAS, Monaco, France). Cytotoxicity levels were expressed as
190 percentage of the viability of the treated Caco-2 cells compared with that of the untreated cells
191 (control).

192

193

194 **2.9. Proteolytic activity**

195 Cells were harvested by centrifugation (8,000 x *g*/10 min, 4°C) after 18 h of growth at
196 37°C. Then, cells were washed three times with PBS (10 mM, pH 7.2) and resuspended in PBS
197 buffer. A volume of 10 µL of each bacterial strain was plated on nutrient agar (Sigma-Aldrich)
198 supplemented with 10% (w/v) skim milk powder (Sigma-Aldrich) sterilized by autoclaving (110
199 °C/ 10 min, 1 atm), and incubated at 37 °C for 72 h. The appearance of a clear zone around the
200 colonies indicates a proteolytic activity (de Albuquerque et al., 2018).

201

202 **2.10. Statistics**

203 All the results presented in this work were obtained from three independent experiments,
204 and the data are expressed as the mean standard deviation. A *P* value of less than 0.05 is
205 considered to be significant using the Student's test.

206

207 **3. Results**

208 **3.1. Absence of PNPase activity alters the bacterial cell envelope structure and reduces** 209 **the cell size**

210 Growth kinetics performed on GM17 medium at 37 °C for 24 hours revealed that *ΔpnpA*
211 mutant strain has a lower growth performance than the WT strain. After 8 hours of growth, the
212 OD_{600nm} of the WT strain was 3.47 while that of the *ΔpnpA* mutant strain was 2.49, i.e. a ratio of
213 1.54 in favor of the WT strain. The measured growth rates were significantly different (*P*=0.001),
214 based on the statistical values and interpretations. As shown in Fig. 1, the complementation of the
215 *ΔpnpA* mutant strain with *pMSP3535:pnpA*, permitted to recover the phenotype of the WT strain

216 as well as its growth performances. Statistically, no difference was observed in terms of growth
217 performances, even on other growth media such as BHI, MRS ($P=0.001$). In addition to the
218 comparison of OD_{600} nm between the WT strain and the $\Delta pnpA$ mutant, we established a colony
219 count (cfu) after 3, 6, 9 and 24 h of growth and therefore concluded to no significant difference
220 ($P> 0.05$). The mean count values (cfu/mL) after 3, 6, 9 and 24 h were 3.17×10^8 , 1.26×10^9 ,
221 2.03×10^9 and 1.40×10^9 for the $\Delta pnpA$ mutant and 2.82×10^8 , 1.40×10^9 , 2.15×10^9 and 1.57×10^9 for
222 the WT strain respectively. After 24 h of growth, $\Delta pnpA$ isogenic mutant strain exhibited clearly
223 smaller colonies than the WT (Fig. 2).

224 Furthermore, TEM analysis performed on both strains after 6 h of growth on GM17
225 medium (Fig. 3) underpinned as expected typical Enterococci diplococci shape, known as rugby
226 ball forms, but not for the $\Delta pnpA$ mutant, which displayed severe alterations on the cell envelope.
227 When several cells were viewed together, we noted that most of $\Delta pnpA$ mutant cells were altered
228 in their cell envelopes and displayed a smaller cell size compared with the WT strain (Fig. 3),
229 arguing on a direct or indirect role of PNPase in this biological pathway.

230

231 **3.2. Mutant deficient in PNPase synthesis has higher ability of adhesion to eukaryotic** 232 **Caco-2 cells than the wild-type strain.**

233 TEM analysis enabled to locate alterations in the cell-envelope of the mutant $\Delta pnpA$, but
234 not in the WT strain. To see whether such alterations could have any role on the adhesion ability
235 to eukaryotic intestinal Caco-2 cells, adhesion assays were conducted with both strains, using two
236 distinct volumes of 200 μ L and 300 μ L but a same cell concentration of $\sim 6.10^9$ cfu/mL. The
237 mutant $\Delta pnpA$ was significantly more adherent to eukaryotic Caco2 compared with the WT
238 strain. The scores of adhesion registered for the mutant strain were $0.22\% \pm 0.015$ (200 μ L), and

239 0.27% \pm 0.028 (300 μ L), whereas those reported for the WT strain were 0.06% \pm 0.010 (200 μ L)
240 and 0.7% \pm 0.010 (300 μ L) (Fig. 4). Thus, the mutant $\Delta pnpA$ was 3.88 (P <0.05) and 3.64 (P <0.05)
241 folds more adherent than the WT with 200 μ L or 300 μ L respectively. In these experiments,
242 whatever the volume used for contact (200 or 300 μ L), no significant difference on the adhesion
243 scores was observed for each strain (P >0.05).

244 To investigate whether the alteration of the cell envelope and adhesion ability of the
245 isogenic mutant could interfere with the cell cytotoxicity, we performed, as shown on Fig. 5,
246 assays measuring the cytotoxicity of the WT and that of the $\Delta pnpA$ mutant against eukaryotic
247 Caco-2 cells. These data confirmed those reported in our previous reports (Caly et al., 2017;
248 Ladjouzi et al., 2020b). Thus, *E. faecalis* 14 WT is not cytotoxic towards eukaryotic Caco-2 cells,
249 as a survival rate of 85.32% was registered after 24 h of contact (Fig. 5). In the absence of
250 PNPase activity, the viability of Caco-2 cells has not significantly changed (P =0.65) as 88.24%
251 of strains were still viable after 24 hours (Fig. 5). These data underpinned a highly adhesion
252 capability of the mutant $\Delta pnpA$ without interfering on the cell cytotoxicity.

253

254 **3.3. *E. faecalis* deficient in PNPase activity is highly proteolytic**

255 The proteolytic activity of the WT and $\Delta pnpA$ mutant was determined to see whether
256 PNPase is involved in such function. Therefore, after 72 h of growth, the $\Delta pnpA$ mutant
257 underpinned intense zones of proteolysis, compared with WT strain (Fig. 6), indicating a putative
258 interaction between PNPase and synthesis of extracellular proteases in *E. faecalis* 14.

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3.4. Transcriptomic analysis revealed a network of deregulated genes

To explain the particular behavior of the $\Delta pnpA$ mutant obtained from *E. faecalis* 14, we performed a global comparative transcriptomic analysis using total RNA isolated from both strains, after 3 h and 6 h of growth in GM17 medium, under semi-aerobic conditions. As depicted on Fig. 7, a total of 244 differently expressed genes (DEGs) were obtained for the $\Delta pnpA$ mutant strain (Table S1). An early deregulation permitted to observe that only after 3 h of growth, expression of 39 genes was up-regulated and that of 16 genes was down-regulated. Nonetheless, after 6 h of growth, the number of DEGs has augmented as 201 genes were up-regulated and only 42 down-regulated (Fig. 7). In light to that, important groups of genes as those involved in different cellular mechanisms resulted to be deregulated in the mutant $\Delta pnpA$, which argue on the pleiotropic role of PNPase (Table S1). In regard to that, we found 186 DEGs; among which 18 were related to stress responses, 94 to different metabolic pathways, 5 to general cell functions and regulations as signal transduction, 36 to translation, 13 to transport, 11 to DNA replication and repair and 2 to other unclassified functions. Moreover, as indicated in Fig. 8, 27 DEGs were found involved in the cell envelope modifications, in particular in the cell wall structure (23 genes), and 4 in the capsule or membrane structure. Notably, 18 other DEGs were related to the bacterial adhesion and biofilm formation (16 genes) and to the proteolysis (2 genes). These collected transcriptomic data are insightful and could partly explain discrepancies in the phenotypes obtained for the $\Delta pnpA$ mutant, especially functions related to bacterial cell wall alterations, cell adhesion and proteolytic activity.

284 4. Discussion

285 mRNA and protein levels are well-regulated in order to continuously satisfy cellular
286 needs. Post-transcriptional regulation relies mRNA stability and protein turnover and modulates
287 the efficiency of mRNA translation into protein. It has become evident that mRNA degradation is
288 a key checkpoint in the hierarchical of regulatory events, and could therefore help to better
289 understand and decrypt global cell response to different environmental growth conditions.

290 In general, mRNA degradation in degradosome of Gram-positive bacteria is initiated by
291 endoribonucleolytic cleavage usually attributed to endoribonuclease Y (RNase Y) leading to 3'
292 tails which are then processed by 3'-5' exoribonucleases, among which PNPase (Cho, 2017).

293 As above-mentioned, PNPase is an ancient 3'-5' exoribonuclease well conserved in both
294 Gram-negative and Gram-positive bacteria. This enzyme has a role in maintaining RNA levels in
295 the cell (Lin-Chao et al., 2007), and also in the mechanisms of RNA processing and degradation
296 (Cameron et al., 2018). Further roles in mRNA homeostasis, tRNA processing and degradation
297 were previously ascribed to PNPase (Li and Deutscher, 1994), supporting thus the idea that this
298 enzyme has a major role in the biology of the bacterial cell.

299 In this work, we establish that the absence of PNPase in *E. faecalis* 14, an isolate from
300 meconium (Al Atya et al., 2015) is characterized by atypical phenotypes suggesting a direct or
301 indirect implication of this 3'-5' exoribonuclease. Briefly, in the $\Delta pnpA$ mutant, we establish a set
302 of modifications, among which clear alteration of the cell-envelope, a high ability of adhesion to
303 Caco-2 eukaryotic cells as well as an enhanced proteolytic activity compared with the WT strain.
304 Further, absence of PNPase seems to have effects on the colony and cell sizes, and cells-
305 organization. Indeed, the $\Delta pnpA$ mutant cells resulted to be smaller in size than those of the WT

306 strain and organized differently than those of the WT strain which are well known as rugby ball.
307 The $\Delta pnpA$ mutant exhibited a better adhesion to Caco-2 cells, without having a toxic effect, and
308 showed as well a better proteolytic activity than the WT strain. All these functions are thought to
309 be directly or indirectly associated with PNPase activity.

310 In this study we also performed a transcriptomic study exploring RNAs isolated from both
311 strains after 3 and 6 h of their growths. A set of genes appeared to be down regulated in the
312 $\Delta pnpA$ mutant and this includes genes associated with transduction, translation, transport, DNA
313 replication, DNA repair, certain metabolic pathways, stress responses, adhesion and biofilm
314 formation, competence and conjugative transfer and other genes related to pathogenesis (See
315 Table S1). In light of this, PNPase was shown to play a role in RNA processing and maturation
316 by modulating the global transcriptome of bacteria like *B. subtilis* and *E. coli*. In direct line,
317 Spanka et al (2021) showed that PNPase can affect the physiology, growth, pigmentation and
318 adaptation of *Rhodobacter sphaeroides* (Spanka et al., 2021). Of note, in *E. coli* a myriad of
319 functions involving a direct or indirect role of PNPase were reported in the literature. These
320 functions include resistance to oxidative stress, UV radiation, and cold acclimation by degrading
321 a large amount of cold shock proteins, transcripts after the cold shock induction (Bonnin and
322 Bouloc, 2015; Rath et al., 2012; Wu et al., 2009), biofilm formation through repression of poly-
323 N-acetylglucosamine (PNAG) (Carzaniga et al., 2012). Furthermore, PNPase activity was also
324 shown to be related to pathogenicity and virulence in Gram-positive and Gram-negative bacteria
325 (Casinhas et al., 2018; Haddad et al., 2009; Numata et al., 2014; Rosenzweig and Chopra, 2013;
326 Sinha et al., 2021).

327 We will now extend this spectrum of functions involving direct or indirect intervention of
328 PNPase to other bacterial models, including Gram-positive bacteria like *B. subtilis*, in which most
329 of the studies on mRNA stability and characterization of ribonucleases were performed.

330 In this bacterium, the PNPase would be involved, at least with DNA repair (Cardenas et
331 al., 2009), and cellular competence (Luttinger et al., 1996). Concerning other Gram-positive
332 bacteria, there is the case of *Lactobacillus*; a genus belonging to the group of lactic bacteria. In
333 fact, the authors Reniero et al (1992) reported a high frequency of aggregation promoting factor
334 (APF)-mediated and conjugative in *Lactobacillus* (Reniero et al., 1992). Interestingly, this factor
335 was highlighted in our transcriptomic study as being over expressed in the $\Delta pnpA$ mutant (Table
336 S1). Similarly, the functions elicited in the literature and presented as having a direct or indirect
337 link with PNPase in Gram-negative and Gram-positive bacteria, also emerge in our
338 transcriptomic study, as being deregulated by the absence of PNPase (Table S1). Of note, our
339 transcriptomic study revealed in the $\Delta pnpA$ mutant no less than 27 up-regulated genes involved in
340 cell envelope synthesis (Table S1 and Fig.8), which may explain in part the phenotypes observed
341 in the $\Delta pnpA$ mutant, which is distinguished by an altered cell envelope, reduced cell size, and
342 unusual cell arrangement (Fig.3). The exploitation of the data from this transcriptomic study also
343 revealed, in the $\Delta pnpA$ mutant, the up-regulation of the *apf* gene coding for the synthesis of the
344 APF protein, whose overproduction causes a drastic alteration of the cell envelope in
345 *Lactobacillus gasseri* 4B2 (Jankovic et al., 2003). This transcriptomic study also revealed a
346 cluster of 16 DEGs involved in adhesion functions and biofilm formation, which could be
347 responsible for the adhesion scores found in the $\Delta pnpA$ mutant strain (Fig.4).

348 In the table S1, a group of genes related to the biofilm formation network was found to be
349 strongly up-regulated in the $\Delta pnpA$ mutant. This group contains the accessory regulator AgrBfs

350 protein, which is involved in the quorum sensing system signal and controls the expression of the
351 virulence factors in *S. aureus* (Zhang et al., 2002). It is noteworthy that gene coding for APF was
352 as well found to be early up-regulated. The APF protein is involved in the conjugation and
353 autoaggregation (Reniero et al., 1992). Remarkably, in the same group of DEGs, there are genes
354 coding for sortase A and cell wall surface anchor family proteins known for their key roles in
355 many infectious diseases (Gaspar et al., 2005; Lalioui et al., 2005). Of note, activation of
356 manganese ABC transporters enables ion import leading to adherence to extracellular matrix
357 (Fenno et al., 1989). Further, other DEGs gathered within this same functional group like those
358 involved in the cell division, complete the molecular biofilm formation network.

359 As depicted on Fig.8, two DEGs were connected to proteolysis processes that were early
360 up-regulated. Thus, the gene coding for a zinc metalloproteinase precursor was up-regulated at 3h
361 and became strongly up-regulated at 6h in the $\Delta pnpA$ mutant strain. Overall, zinc
362 metalloproteases belong to microbial proteases, which are predominantly extracellular involved
363 in the virulence (Häse and Finkelstein, 1993). Similarly, an up-regulation was observed for the
364 gene coding for a serine proteinase of V8 family performing proteinase activity (Dancer et al.,
365 1990). Another early-regulated gene was that coding for an extracellular protein, suggesting that
366 the $\Delta pnpA$ mutant strain increases several biotic and/or abiotic interactions (Dalbey and Kuhn,
367 2012), probably involved in defense or virulence functions (Lee and Schneewind, 2001; Ma and
368 Guttman, 2008). Taken together, these up-regulated proteinases could explain the enhanced
369 proteolytic activity observed for the $\Delta pnpA$ mutant strain (Fig.6). Indeed, PNPase was reported to
370 be important in the regulation of small RNAs that control the outer membrane proteins
371 expression (Andrade and Arraiano, 2008).

372 It was reported that ribonuclease J1 is essential in *E. faecalis* (Salze et al., 2020). In this
373 transcriptomic study, we observed that the gene encoding this enzyme is overexpressed in the
374 mutant after 6 hours of growth, most likely indicating a compensatory effect to compensate for
375 the absence of PNPase. Autoregulation of RNase gene expression exploit enzymatic activity and
376 act at the level of mRNA stability in a Gram-positive bacterium (Condon and Bechhofer, 2011),
377 and presumably, the same mechanism is occurring in *E. faecalis* 14.

378 5. Conclusion

379 In this study, we constructed a mutant deficient in PNPase synthesis in *E. faecalis* 14; a
380 strain formerly isolated from meconium. The transcriptomic study enabled first snapshot on the
381 genes or the network of genes that are deregulated in the absence of the PNPase. However, from
382 a physiological point of view, it is clear that the absence of this major 3'-5' exoribonuclease has
383 an impact on the physiology and cellular structure of the bacterium. In addition to the alteration
384 of the cell envelope, we have highlighted the reduced size of the cells and colonies in the $\Delta pnpA$
385 mutant strain than in the WT strain. Similarly, the $\Delta pnpA$ mutant strain has a high proteolytic
386 activity and a better ability to adhere to Caco2 cells than the WT. Therefore, all these observed
387 effects involve directly or indirectly the PNPase activity in *E. faecalis* 14.

388

389 **Conflict of interest:** The authors declare no competing interests

390 **Author contributions:** Conceptualization and experiment design: DD, RL, MD and ADL. RL
391 and MD performed the experiments and ADL carried out the transcriptomic analysis. DD, RL,
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402

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576 **Figure legends**

577 **Fig. 1:** Growth curves of *E. faecalis* WT (●), $\Delta pnpA$ mutant (■) and the $\Delta pnpA$ -complemented
578 (▲) strains grown in GM17 medium. The standard deviations are presented by vertical bars. The
579 asterisk (*) indicates that the growth rate is significantly different from that of the WT strain
580 using the student test ($p < 0.05$).

581 **Fig. 2.** Macroscopic appearance and size of *E. faecalis* WT, $\Delta pnpA$ mutant and the $\Delta pnpA$ -
582 complemented colonies on the same BHI agar plate.

583 **Fig. 3.** Transmission electron microscopy (TEM) micrographs of *E. faecalis* WT and its $\Delta pnpA$
584 mutant strain. Arrows indicate the main alterations on cell wall of the $\Delta pnpA$ mutant

585 **Fig. 4.** Adhesion of *E. faecalis* WT and its $\Delta pnpA$ mutant strains to Caco-2 cells after 2h30 mn
586 of contact. The contact was performed using two volumes 200 μ L and 300 μ L (6.10^9 cfu.mL⁻¹).
587 The vertical bars represent the standard deviations. The asterisk (*) indicates a significantly
588 different growth rate ($p < 0.05$) between the $\Delta pnpA$ mutant and the WT.

589 **Fig. 5.** Cytotoxic effect of *E. faecalis* 14 WT and its $\Delta pnpA$ mutant strains on Caco-2 cells after
590 24 h of contact. Three independent experiments were performed and the means of data are
591 represented. Means and standard bars are shown. “ns” denotes: not significant

592 **Fig. 6.** Proteolytic activity of *E. faecalis* 14 WT and its $\Delta pnpA$ after 72 h of growth on milk
593 agar. A representative profile of three independent experiments is presented.

594 **Fig. 7.** Venn diagram representing the number of up- and down-regulated DEGs in $\Delta pnpA$ strain
595 of *E. faecalis* at 3h and 6h of culture respectively.

596 **Fig. 8.** Gene expression profiles of DEGs involved in cell envelope modifications and
597 pathogenesis in $\Delta pnpA$ strain of *E. faecalis*. Log₂ FC of individual $\Delta pnpA$ (P1-P3) vs. mean of

598 WT were represented for 3h and 6h of culture respectively. Functional groups are indicated in
599 the right of the picture.

600 **Table 1.** Bacterial strains and plasmids used in this study.

Designation	Relevant characteristics	Reference
Strain		
Enterococci		
Enterococcus faecalis 14	Wild-type strain isolated from meconium	(Al Atya et al., 2015)
14 ΔpnpA	<i>E. faecalis</i> 14 <i>pnpA</i> deletion mutant	This study
14 ΔpnpA -C	<i>E. faecalis</i> 14 Δ <i>pnpA</i> harboring pMSP3535: <i>pnpA</i> complemented strain	This study
<i>E. coli</i>		
XL-1 Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 Lac</i> [F' <i>proAB lacI</i> λ ZAM15Tn10 (Tet ^R)]	Stratagene
JM109	<i>recA1. endA1, gyrA96, thi. hsdR17, supE44, relA1, I-, A(iac-proAB), [F', traD36, proAB, iac]λZAM15]</i>	(Yanisch-Perron et al., 1985)
Plasmids		
pLT06	<i>lacZ</i> , P- <i>pheS</i> from pCJK47, <i>Cm</i> from pGB354, <i>orfB</i> , <i>orfC</i> , <i>repA</i> (<i>Ts</i>), <i>orfD</i> from pCASPER	(Thurlow et al., 2009)
pLT06ΔpnpA	pLT06 derivative carrying a 1.91 kb DNA fragment from <i>E. faecalis</i> 14 harboring mutated <i>pnpA</i> gene	This study
pMSP3535	Em ^R , <i>nisR</i> , <i>nisK</i> <i>PnisA</i> (nisin-inducible promoter)	(Bryan et al., 2000)
pMSP3535:<i>pnpA</i>	pMSP3535 derivative carrying a 2.36 kb DNA fragment from <i>E. faecalis</i> 14 with <i>pnpA</i> gene	This study
pGEM-t Easy	Ap ^R cloning vector	Promega
pGEM-t EasyΔpnpA	pGEM-t Easy derivative carrying a 1.91 kb DNA fragment from <i>E. faecalis</i> 14 harboring mutated <i>pnpA</i> gene	This study

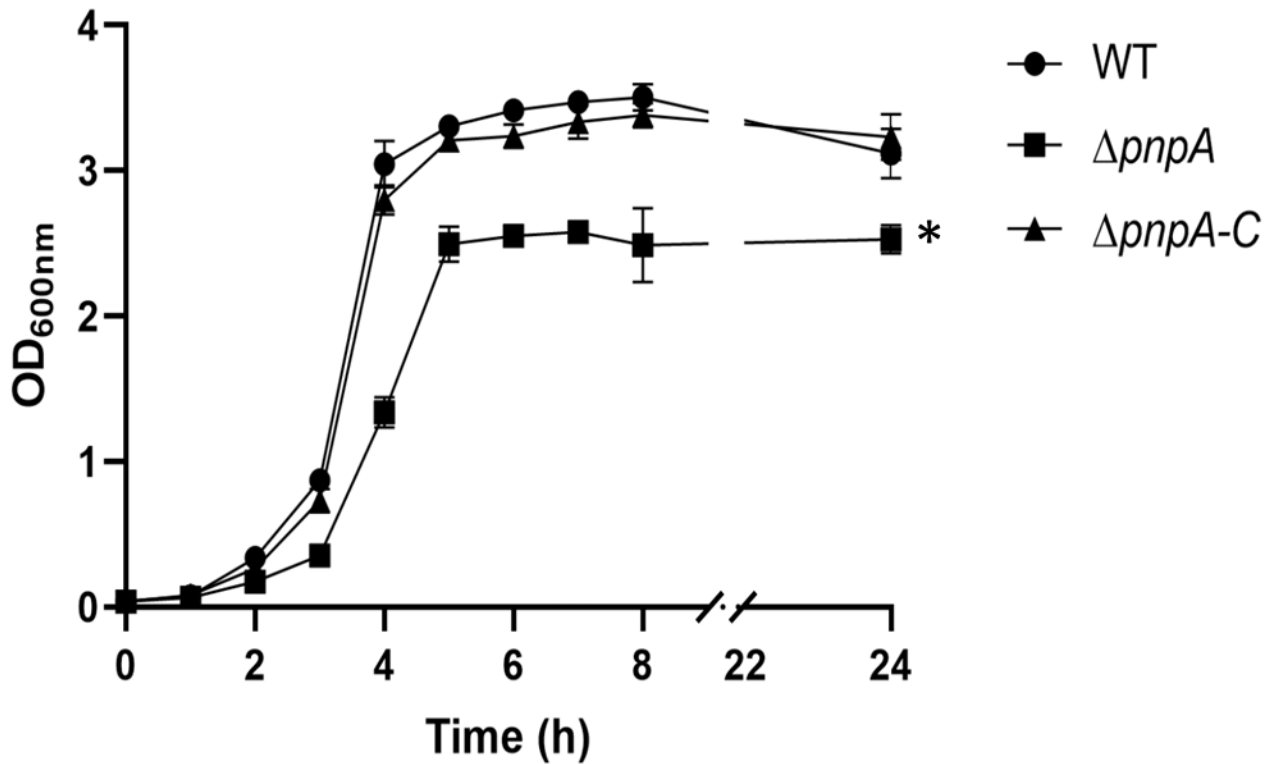
601 *Em*, erythromycin; *Ap*, ampicillin; *Tet*, tetracycline; *Cm*, chloramphenicol; *bgaB*, Beta-galactosidase; *R*,
602 resistant; *s*, sensitive; *Ts*, thermosensitive; *nis*, nisin

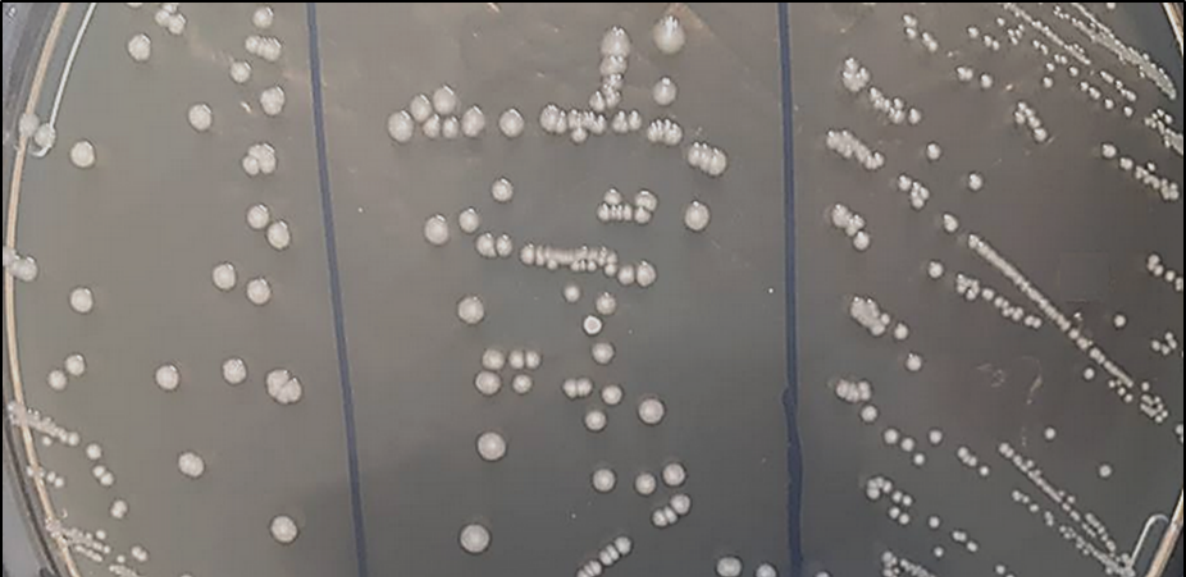
603 **Table 2.** Oligonucleotides used in this study.

Name	Sequence 5'----->3'	The use for
pnpA-1F	ACCCTCGAGTTCAATGCGGACAAC <u>TTTTG</u>	amplification of <i>pnpA</i> up-stream fragment
pnpA-2R	CCAACGTTT <u>AGGATTACGTCGCGGATGGCCGTAAATTAGAC</u>	
pnpA-3F	GTCTAATTTACGGCCATCCGCGACGTAATCCTAAACGTTGG	amplification of <i>pnpA</i> down-stream fragment
pnpA-4R	TGTGAATTCCGCCAGCCATGTCAAATA	
pnpA-5F	GCGCAAATACTTGCTTGTGA	Internal primers; verification of the plasmid integration
pnpA-6R	AAATCGGTCACCGTCGTAAC	
pnpA-7F	ATGGATCAAGCCATCTTTGC	External primers, screening of the mutants
pnpA-8R	AAAAAGTGGCCACAATGGTC	
pnpA-CompF (<i>Pst</i> I)	ATTAA <u>ACTGCAGG</u> GAAGCTTCCTTGGTTTTCG	Complementation of $\Delta pnpA$ mutant
pnpA-CompR (<i>Bam</i> HI)	ATTAA <u>AGGATCCG</u> GAGCAGCAACGACTTGTTAC	
PU	GTAAAACGACGGCCAGT	Cloning verification in pGEM-t Easy plasmid
PR	CAGGAAACAGCTATGAC	
PMSP3535-F	GATACAATGATTTTCGTTTCGAAGG	Cloning verification in pMSM3535 plasmid
PMSP3535-R	GCTTATCGAAATTAATACGACTCAC	
ORIf	CAATAATCGCATCCGATTGCA	Cloning verification in pLT06 plasmid
KS05seqR	CCTATTATACCATATTTTGGAC	

604 *Underlined sequences correspond to the recognition sites of the restriction endonucleases mentioned in
605 brackets in the primer name.

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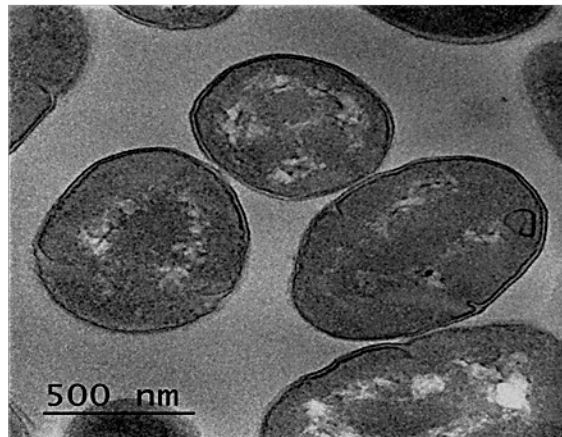
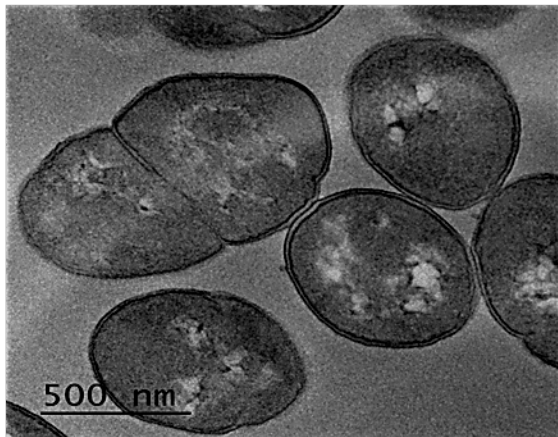
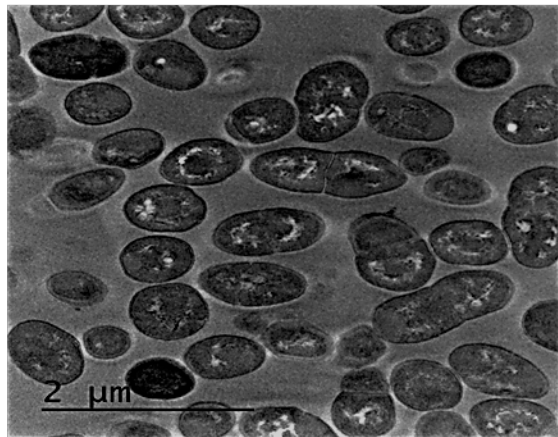


WT

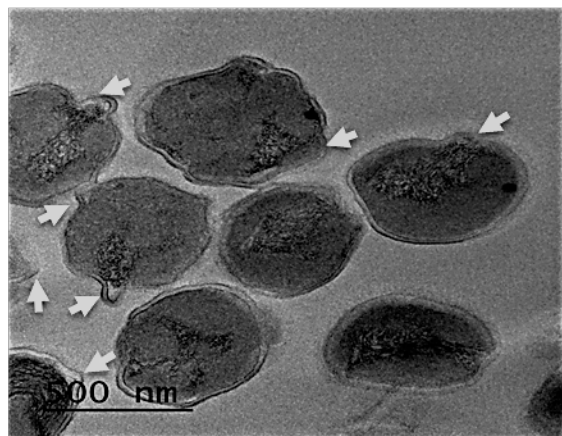
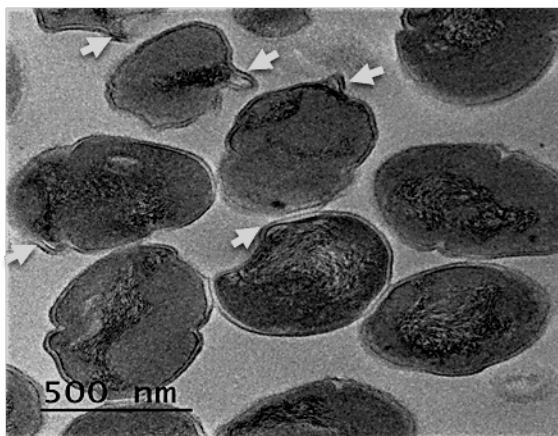
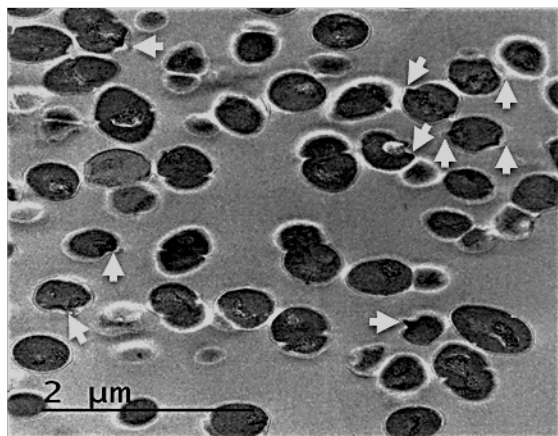
$\Delta prpA-C$

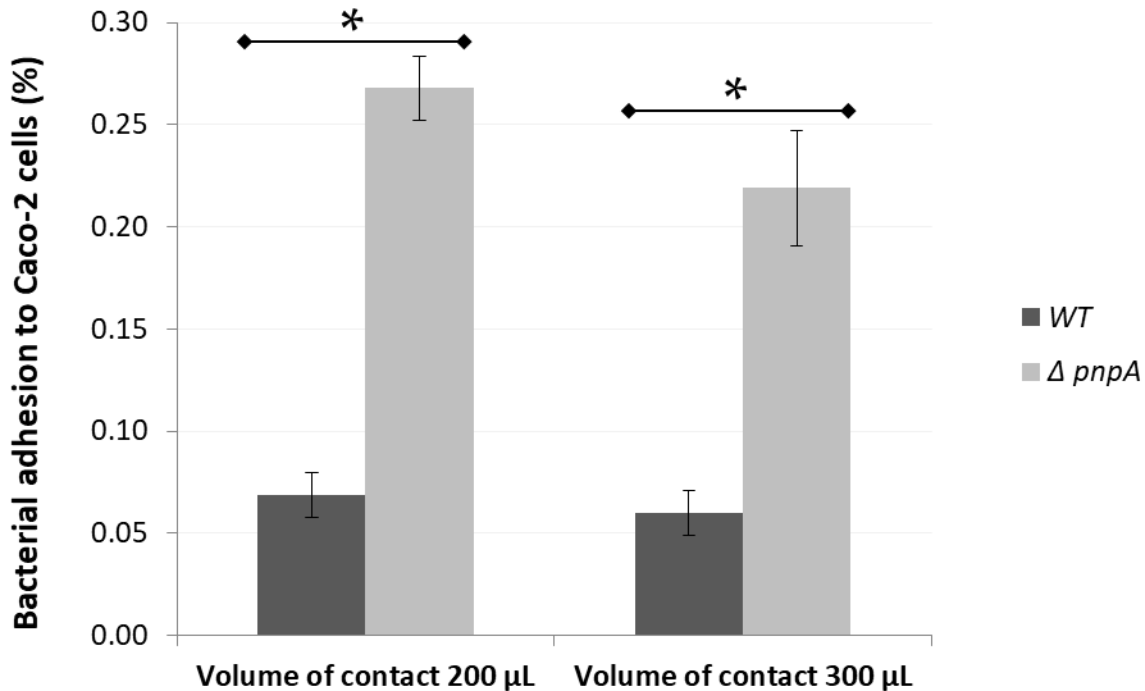
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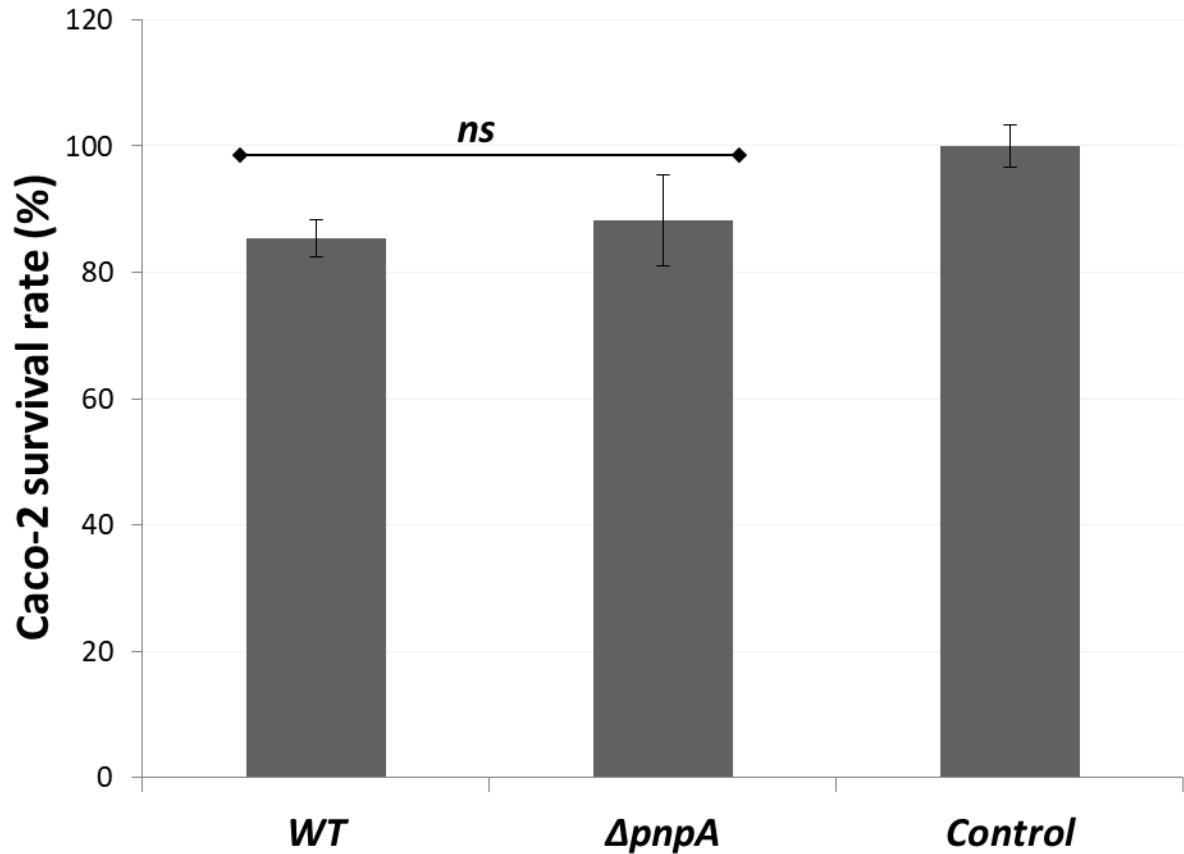
E. faecalis WT

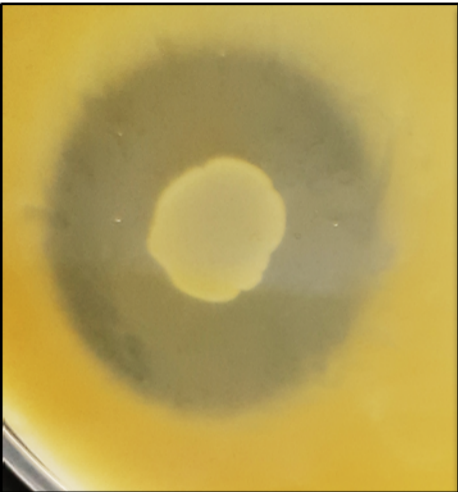


E. faecalis Δ *prpA*

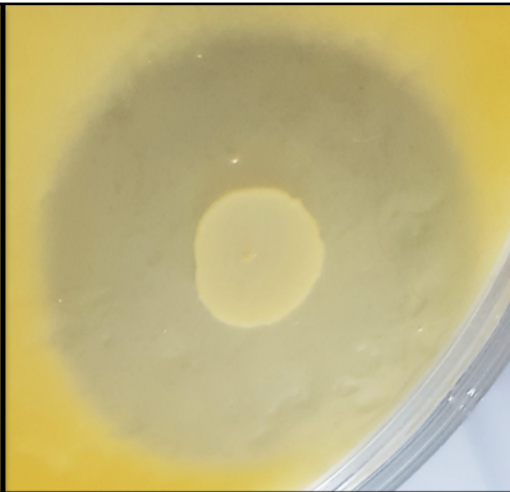




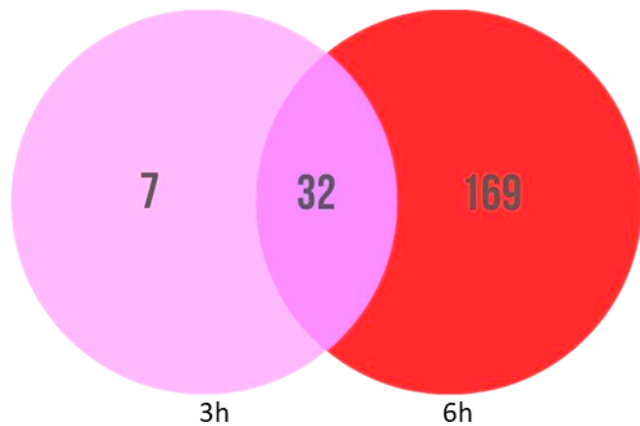




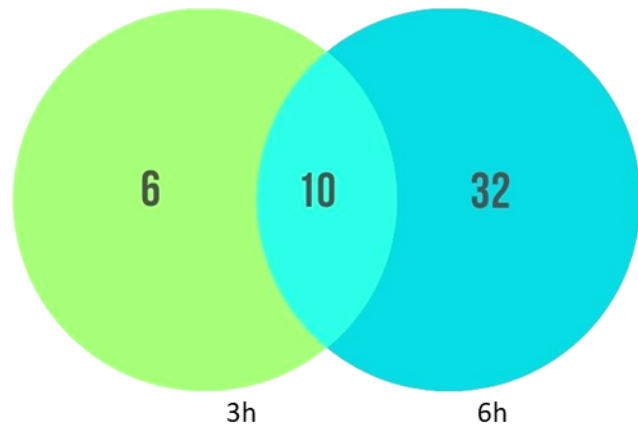
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$\Delta prpA$



Up-regulated genes



Down-regulated genes

