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**Short Communication**

# A tunable heterologous phosphatidylcholine biosynthetic system reveals distinct phosphatidylcholine requirements for strong and weak acid responses in *Acetobacter pasteurianus*

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**Running head:**

PC and acid response in *A. pasteurianus*

**Summary**

Acetic acid bacteria, including the genera *Acetobacter* and *Komagataeibacter*, utilize phosphatidylcholine (PC) as a major membrane component, which plays an important role in their membrane physiology. However, the significance of PC abundance within the membrane remains speculative. Here, we constructed a mutant strain of *A. pasteurianus* through genome modification, enabling choline-dependent PC production. This mutant exhibited different dependencies on PC levels in response to strong and weak acid stress, requiring higher PC levels under strong acid stress but lower levels under weak acid stress. These findings provide new insights into the membrane-based mechanisms of acid-stress response in *A. pasteurianus*.

**Keywords:**

acetic acid bacteria; *Acetobacter pasteurianus*; phosphatidylcholine; acid stress; stress response

Phosphatidylcholine (PC), the most abundant phospholipid (PL) in eukaryotes, is a bilayer-forming component that governs membrane integrity. Although PC was previously thought to exist only in eukaryotes, it is now estimated that at least 15% of bacterial species synthesize PC (Geiger et al., 2013). Levels of PC range from trace amounts (as in *Pseudomonas aeruginosa*) to over 70% of total PLs (as in *Acetobacter aceti*) (Hanada et al., 2001; Wilderman et al., 2002). Recent studies have highlighted the physiological relevance of bacterial PC. For example, PC supports the symbiotic

performance of nitrogen-fixing bacteria such as *Sinorhizobium meliloti* (Geiger et al., 2021) and is involved in the virulence of pathogenic bacteria such as *Agrobacterium tumefaciens* (Wessel et al., 2006). Omics-based approaches have provided insights into the effects of PC deficiency on the proteome and transcriptome (Geiger et al., 2021; Klüsener et al., 2010), but the detailed molecular basis of PC function within the membrane remains unclear. One difficulty in elucidating these mechanisms is that PC deficiency affects not only membrane protein function but also overall membrane organization.

Acetic acid bacteria (AAB) are known for their ability to oxidize sugars and alcohols to organic acids (Adachi and Yakushi, 2016). Specifically, *Acetobacter* and *Komagataeibacter* species oxidize ethanol to produce acetic acid (Nakano and Ebisuya, 2016). Notably, AAB maintain high levels of PC within their membranes, and PC has been considered to contribute to their remarkable acid resistance (Nakano and Ebisuya, 2016). In support of this, it was reported that a PC-deficient mutant of *A. aceti* is highly sensitive to acetic acid (Hanada et al., 2001). However, the molecular mechanism through which PC regulates the acid stress response is poorly understood. High levels of PC in membranes are proposed to enhance membrane stability and to function as a physicochemical barrier against acetic acid in *A. pasteurianus* (Han et al., 2020). Nevertheless, the exact relationship between membrane PC levels, membrane stability, and acid stress response is challenging to determine. One difficulty is that AAB synthesize PC from phosphatidylethanolamine (PE, one of the other major PLs in AAB), complicating the precise control of PC production and the isolation of its effects. To overcome these obstacles, we engineered a mutant strain derived from *A. pasteurianus* SKU1108 (Saeki et al., 1997), for which molecular genetic tools have been established

66 (Yakushi et al., 2018). This engineered mutant strain enables choline-dependent, tunable
67 PC production, and therefore provides a unique opportunity to study how variations in
68 membrane PC levels affect membrane properties and stress responses.

69 AAB synthesize PC via the methylation pathway, in which PE is stepwise converted
70 into PC by a single PE *N*-methyltransferase (PmtA) (Hanada et al., 2001). Previously, we
71 constructed a *pmtA*-deletion mutant (Δ*pmtA*) of *A. pasteurianus* SKU1108, using the
72 smooth (S) variant as the parental strain, via homologous recombination with the suicide
73 pKOS6b plasmid (Kostner et al., 2013; Toyotake et al., 2026). We also demonstrated that
74 heterologously expressed PC synthase (Pcs), which catalyzes the condensation of CDP-
75 diacylglycerol (DAG) with exogenous choline, was functional in this mutant. We
76 therefore applied the same pKOS6b-based recombination system to introduce the gene
77 encoding Pcs derived from *P. aeruginosa* into the *pmtA* deletion locus on the Δ*pmtA*
78 genome (Fig. 1A) (see Supplementary Methods for details). The resulting mutant strain
79 was named Δ*pmtA+pcs*. The integration of the *pcs* gene was confirmed using PCR with
80 primer pairs specific to the wild-type and Δ*pmtA+pcs* genomes (Fig. 1B); the intended
81 single band appeared only when primer pairs specific to each genome were used (Fig.
82 1C), confirming that the *pcs* gene was inserted into the expected locus on the genome.
83 The Δ*pmtA+pcs* genome was further validated by gene sequencing. These results
84 demonstrated that the *pcs* gene in the Δ*pmtA+pcs* strain was successfully located under
85 the control of the native *pmtA* promoter.

86 In this study, we used the S variant of *A. pasteurianus* SKU1108 as the parental
87 strain for all experiments. To evaluate choline-dependent PC production, we analyzed the
88 PL class composition of the parental, Δ*pmtA*, and Δ*pmtA+pcs* strains grown in choline-
89 containing medium (Fig. 2). The cells were cultivated at 30 °C in YPG medium [0.5%

(w/v) yeast extract D-3H (Shiotani MS, Hyogo, Japan), 0.5% (w/v) Hipolypepton (Shiotani MS), and 1% (w/v) glycerol] supplemented with 0–50 mM choline chloride (C7527; Sigma-Aldrich, St. Louis, MO, USA) for 24 hours. The cells were harvested by centrifugation. Total membrane PLs were extracted from the resulting cell pellets using the method of Bligh and Dyer (1959) and then developed on silica gel 60 thin-layer chromatography (TLC) plates (Supelco, Bellefonte, PA, USA) using a chloroform–methanol–acetic acid (6.5:2.5:1, v/v/v) solvent system. The TLC plates were stained with primulin solution [0.005% (w/v) primulin in acetone–water (4:1, v/v)]. Each PL class was identified by comigration with standard PLs (Avanti Polar Lipid, Alabaster, AL, USA). The bands corresponding to each PL class was identified under UV light at 365 nm, the silica gel was scraped off, and then PLs were quantified using a phosphate assay (Rouser et al., 1966) with $KH_2PO_4$ as the phosphate standard.

As shown in Fig. 2, the PL class composition of parental and Δ*pmtA* strains was generally unaffected by choline supplementation. Under all conditions tested, the relative amount of PC was maintained between 50% and 53% in the parental strain and undetectable in the Δ*pmtA* strain. However, the relative amounts of PE and phosphatidylglycerol (PG) increased in the Δ*pmtA* strain. Low levels of cardiolipin (CL) were present in both strains. In the Δ*pmtA*+*pcs* strain, PC production increased in a choline dose-dependent manner from 0 to 0.6 mM, and then plateaued above 0.6 mM—even when 50 mM choline was added, there was only a slight increase in PC production from 35.8% to 37.6%. These results indicated that there is an upper limit to PC production in the Δ*pmtA*+*pcs* strain, for which there are several possible reasons. First, choline uptake or Pcs-dependent PC production may become saturated at high choline concentrations. Second, metabolic competition between the exogenous Pcs-dependent PC

114 biosynthesis pathway and the endogenous biosynthesis pathways of other PLs may occur
115 for the common precursor CDP-DAG (Jennings and Epand, 2020). It should be noted that
116 a PC level of 4.5% was detected in the Δ*pmtA+pcs* strain, even in the absence of choline
117 supplementation. Given that YPG medium already contains some choline salts, it is
118 reasonable to assume that Pcs was able to utilize these salts. Collectively, the results show
119 that the Δ*pmtA+pcs* strain could produce PC using choline as a substrate, with a
120 maximum level of nearly 38%, approximately 12%–15% lower than the parental level.
121 However, this was the highest level compared with other PLs in that mutant. Furthermore,
122 the relative amounts of PE and PG fluctuated synchronously with those of PC. Hence, by
123 adjusting choline concentrations, the PL class composition balance in the Δ*pmtA+pcs*
124 strain can be restored to almost the parental level. This can bridge the phenotypic gap
125 between the parental and Δ*pmtA* strains.

126 To clarify the structural details of membrane PLs, we analyzed the individual
127 molecular species within each PL class in the parental, Δ*pmtA*, and Δ*pmtA+pcs* strains
128 grown in choline-supplemented medium (Fig. S1). Each class was separated as described
129 above, extracted from the scraped silica gel, and analyzed using electrospray ionization-
130 tandem mass spectrometry (ESI-MS/MS) on a triple-quadrupole Sciex API 3000$^{TM}$
131 LC/MS/MS System (Sciex, Concord, Ontario, Canada) in negative mode, as previously
132 described (Toyotake et al., 2018). In all strains, the main component was 36:2 species
133 containing two unsaturated fatty acids (18:1/18:1), followed by 37:2 species containing
134 one cyclopropane fatty acid (18:1/19:0c). The relative amounts of PL species were largely
135 unaffected by choline supplementation. However, a notable exception was observed in
136 the Δ*pmtA+pcs* strain, where the relative amount of PC 36:2 was higher than in the other
137 strains, whereas that of PC 37:2 was lower, especially under low-choline supplementation

conditions (0 mM and 0.05 mM). This suggested that Pcs prefers the production of PC 36:2. However, the extent to which this preference affects the phenotype of this mutant remains to be clarified.

Subsequently, to determine how alterations in membrane PC levels affect the physiological responses of *A. pasteurianus*, we investigated growth and acid stress response of the parental, Δ*pmtA*, and Δ*pmtA+pcs* strains under various choline concentrations (Fig. S2 and Fig. 3). Compared with the parental strain, growth of the Δ*pmtA* strain was impaired; this was particularly pronounced at 50 mM choline. In contrast, growth of the Δ*pmtA+pcs* strain partially recovered without choline supplementation, but almost completely recovered when choline concentrations exceeded 0.05 mM (Fig. S2). Given that the relative amount of PC in the Δ*pmtA+pcs* and parental strains grown in 0.05 mM choline was 14.0% and 52.3%, respectively (Fig. 2), it is possible that PC levels as low as approximately one-fourth of the parental level are sufficient to maintain normal growth. We previously reported that ~4% PC is sufficient to restore normal growth even without choline supplementation when Pcs was expressed from a plasmid (Toyotake et al., 2026). The difference in PC requirement observed here may be related to differences in the mode of Pcs expression. Nevertheless, our results indicate that PC can support normal growth even at levels substantially lower than those in the parental strain. However, it could be argued that the growth phenotypes are affected not only by PC levels but also by changes in the overall membrane PL content. To address this possibility, we measured the phosphorus content of membrane lipids in the strains grown in YPG medium. When normalized to total lipid mass, phosphorus levels were comparable among the strains: 0.46 ± 0.02 nmol P/μg for the parental strain, 0.46 ± 0.02 for the Δ*pmtA* strain, and 0.46 ± 0.01 for the Δ*pmtA+pcs* strain (mean ± SD, $n = 3$). These

results indicate that alterations in membrane PC levels do not change the overall membrane PL content. Furthermore, they suggest that compensatory changes under PC depletion conditions, such as an increase in PG, are insufficient to maintain normal growth.

To examine the acid stress response, we performed a spot assay. Cells were cultivated in YPG medium for 24 hours, harvested, and resuspended in YPG medium to an optical density at 600 nm ($OD_{600}$) of 1. Ten-fold serial dilutions of the suspension were prepared in YPG medium, and 3 µL of each dilution was spotted onto acidified YPG agar (1%) plates adjusted to pH 3.8 or 3.5 using either acetic acid or hydrochloric acid (HCl) (see Supplementary Methods for details). Choline chloride was added to the medium at increasing concentrations, and the plates were incubated at 30 °C until colony formation was observed (Fig. 3).

Under acetic acid stress at pH 3.8, the Δ*pmtA* strain exhibited severe growth defects compared with the parental strain. Growth of the Δ*pmtA+pcs* strain was also impaired without choline supplementation, although this impairment was alleviated with low choline concentrations (≥30 µM) and fully recovered with 100 µM choline (Fig. 3A, pH 3.8). At the same pH under HCl stress, a slight growth defect was observed in the Δ*pmtA* strain. In the absence of choline supplementation, a similarly mild growth defect was observed in the Δ*pmtA+pcs* strain, but growth was almost completely recovered with low choline concentrations (≥10 µM) (Fig. 3B, pH 3.8). When the pH was reduced to 3.5, the Δ*pmtA+pcs* strain required markedly different choline concentrations under acetic acid and HCl stress. Under acetic acid stress at pH 3.5, the growth defect of the Δ*pmtA+pcs* strain was alleviated by choline concentrations of ≥40 µM, whereas 200 µM was required for complete restoration (Fig. 3A, pH 3.5). In contrast, under HCl stress at pH 3.5, growth

of the Δ*pmtA+pcs* strain was more severely impacted than at pH 3.8; choline concentrations of ≥400 μM and 1,000 μM were required for alleviation and complete restoration, respectively (Fig. 3B, pH 3.5).

The choline concentration required to alleviate stress likely reflects the membrane PC levels needed for that stress response. The PL class composition analysis suggested that PC production in the Δ*pmtA+pcs* strain did not reach the parental level in the presence of 200 μM choline (Fig. 2). However, this concentration completely restored the Δ*pmtA+pcs* growth under acetic acid stress (Fig. 3A). In addition, when the acetic acid stress was alleviated from pH 3.5 to 3.8, the Δ*pmtA+pcs* growth remained defective, but the choline concentration required to recover its growth decreased (Fig. 3A). Taken together, these observations suggest that low levels of PC are sufficient to support an appropriate response to acetic acid stress. Acetic acid diffuses across the membrane in its undissociated form, leading to a rapid decrease in intracellular pH. Membrane-associated acetic acid efflux systems have been proposed to respond to this stress. In *A. aceti*, membrane proteome analysis identified a putative ABC transporter (Nakano et al, 2006). In *A. pasteurianus*, a proton-driven secondary transport system was inferred from biochemical analysis (Matsushita et al., 2005). It is conceivable that low levels of PC may either directly or indirectly support such systems. To test this hypothesis, investigations into how low levels of PC affect the membrane proteome, or the identification of membrane proteins that interact with PC, should be pursued. However, it should be noted that *A. pasteurianus* undergoes metabolic changes when exposed to acetic acid for a period, enabling the consumption of acetic acid (Kanchanarach et al., 2010). This metabolic change may support the growth of cells that survived transient acetic acid exposure. In this context, another possible scenario is that low levels of PC stabilize the

membrane and prevent rapid cell death. Therefore, it is necessary to determine the extent to which low levels of PC can restore membrane properties, such as stability and fluidity, compared with parental levels. It should also be considered that low levels of PC may be locally enriched and function within a heterogeneous membrane. Nevertheless, our findings challenge the conventional perspective and support our previous model (Toyotake et al., 2026), suggesting that PC function in *A. pasteurianus* may not necessarily depend on its quantitative abundance within the membrane.

Under moderate HCl stress at pH 3.8, a slight defect in growth was recovered with low levels of PC (Fig. 3B). PC may have some involvement in antiporter systems that participate in pH homeostasis (Soemphol et al., 2015). Alternatively, membrane stability that can be restored at low levels of PC may be sufficient to alleviate such mild acid stress. Notably, despite comparable external proton strength at pH 3.5, substantially higher levels of PC were required to alleviate HCl stress than acetic acid stress (Fig. 3, see pH 3.5 conditions). Strong acids, such as HCl, dissociate in medium and continuously impose proton stress on the membrane from the outside. In addition, *A. pasteurianus* cannot alleviate HCl stress metabolically. Higher levels of PC may therefore be necessary to sufficiently maintain membrane stability under such persistent proton stress.

In conclusion, using our engineered mutant strain with tunable PC production, we found that *A. pasteurianus* exhibits distinct responses to strong acid (HCl) and weak acid (acetic acid) based on PC levels in the membrane. These findings provide new insights into the unique membrane-based acid stress response mechanisms of this bacterium.

## Supplementary information

Supplementary information is available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

## Author contributions

MRHK conducted the experiments and drafted the manuscript. YT conceived the study, secured funding, designed and conducted the experiments, performed data analysis, and wrote and revised the manuscript. MW provided resources.


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**Figure captions**

**Fig. 1. Genetic engineering of the choline-dependent PC producing mutant (Δ*pmtA*+*pcs*).**

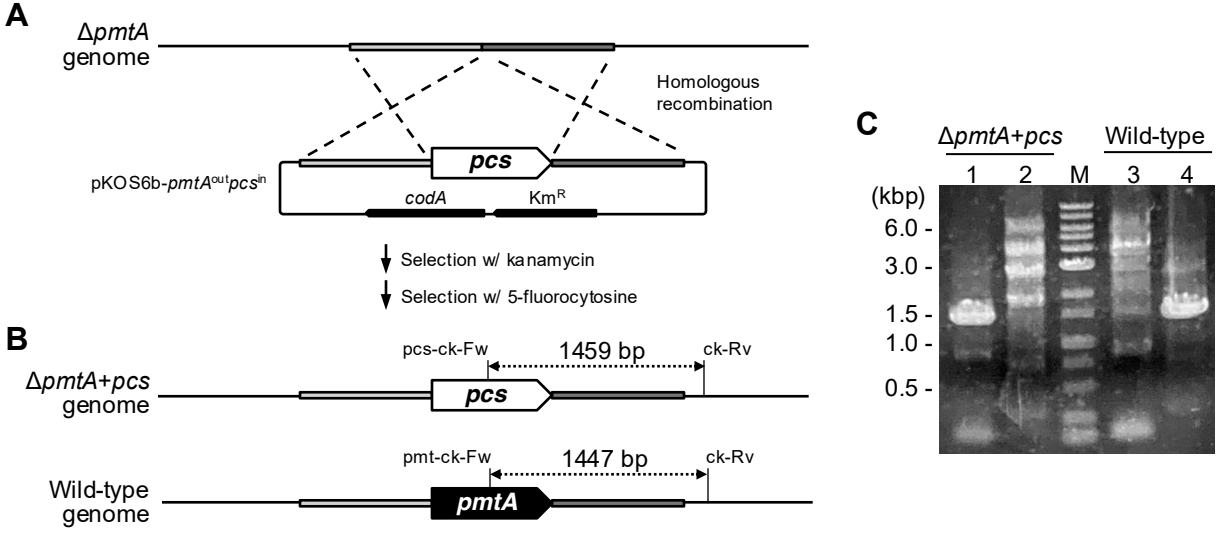
A. The plasmid pKOS6b-*pmtA*$^{out}$*pcs*$^{in}$ harboring the upstream (light gray) and downstream (dark gray) regions of *pmtA* connected by *pcs* was introduced into the Δ*pmtA* strain. Through homologous recombination between pKOS6b-*pmtA*$^{out}$*pcs*$^{in}$ and the genome, a plasmid-integrated kanamycin-resistant recombinant was obtained. A second homologous recombination between two corresponding regions of the genome in the presence of 5-fluorosytosine yielded a choline-dependent PC-producing mutant named Δ*pmtA*+*pcs*, in which *pcs* was inserted into the designated location on the genome. B. The genomic difference between Δ*pmtA*+*pcs* and the wild-type is depicted. The locations of *pcs* in Δ*pmtA*+*pcs* and *pmtA* in the wild-type are identical. The primers used for PCR are indicated as pmt-ck-Fw, pcs-ck-Fw, and ck-Rv. C. PCR-amplified DNA from the Δ*pmtA*+*pcs* genome using pcs-ck-Fw and ck-Rv (lane 1) or pmt-ck-Fw and ck-Rv (lane 2) and from the wild-type genome using pcs-ck-Fw and ck-Rv (lane 3) or pmt-ck-Fw and ck-Rv (lane 4). The DNA size maker is placed in the center (lane M). The genome and plasmid are not depicted to scale.

**Fig. 2. Choline-dependent PC production in the Δ*pmtA*+*pcs* strain**.

Total membrane PLs were extracted from the parental, Δ*pmtA*, and Δ*pmtA*+*pcs* strains grown for 24 hours in YPG medium supplemented with 0, 0.05, 0.1, 0.6, or 50 mM choline. Individual classes of PL were separated using TLC and quantified using a

phosphate assay. The mean (± SD) relative amounts (mol%) of PL classes are shown (*n* = 3).

**Fig. 3. The effect of different PC levels on stress responses to acidic conditions.** Serial dilutions of parental, Δ*pmtA*, and Δ*pmtA+pcs* strains were spot-incubated on acidified YPG agar plates adjusted to pH 3.8 or 3.5 by the addition of acetic acid (A) or HCl (B). Choline was added at final concentrations ranging from 0 to 200 μM in acetic acid-containing plates and from 0 to 1,000 μM in HCl-containing plates. YPG plates (pH 6) without supplementation served as the standard. The plates were incubated at 30 °C for 2–3 days until colony formation was observed.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ΔpmtA genome
Homologous recombination
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pcs
codA
KmR
Selection w/ kanamycin
Selection w/ 5-fluorocytosine
B
ΔpmtA+pcs genome
pcs-ck-Fw
1459 bp
ck-Rv
pcs
Wild-type genome
pmt-ck-Fw
1447 bp
ck-Rv
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Wild-type
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3.0
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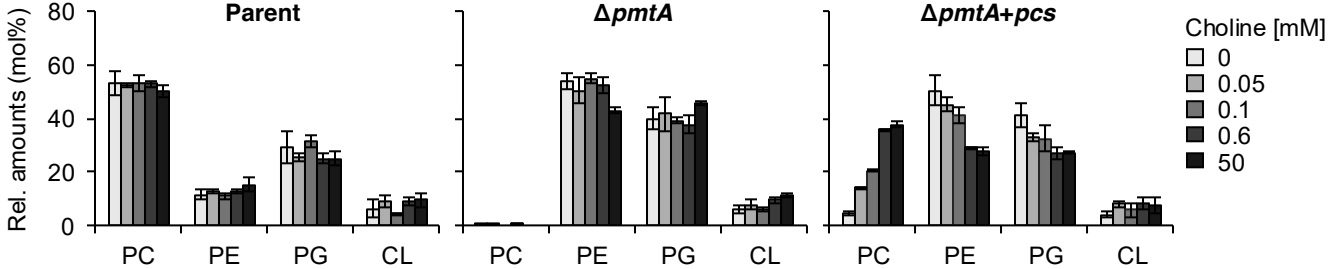
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ΔpmtA+pcs
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20
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PG
CL
Choline [mM]
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0.1
0.6
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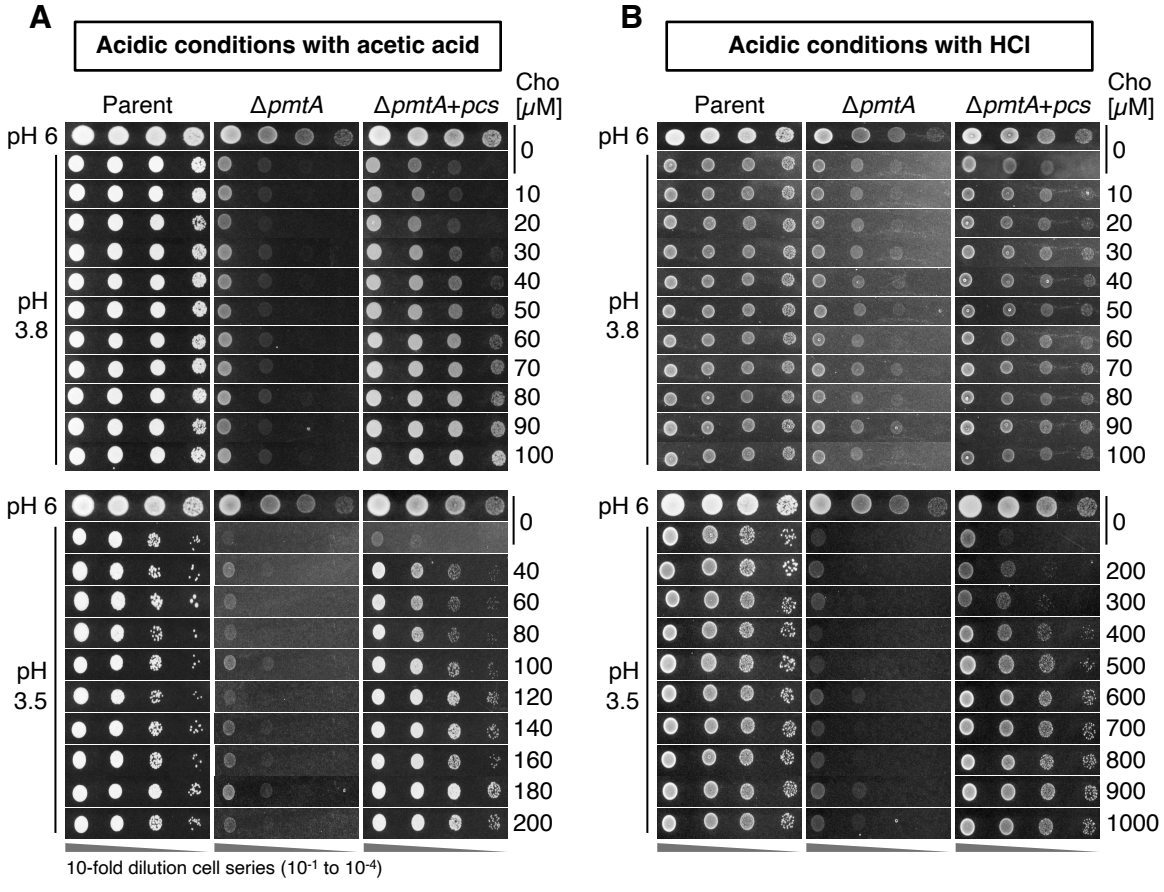
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Acidic conditions with acetic acid
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