Highly conserved nucleotide phosphatase essential for membrane lipid homeostasis in *Streptococcus pneumoniae*

Kuipers, Kirsten; Gallay, Clement; Martinek, Vaclav; Rohde, Manfred; Martinkova, Marketa; van der Beek, Samantha L.; Jong, Wouter S. P.; Venselaar, Hanka; Zomer, Aldert; Bootsma, Hester

*Published in:*
Molecular Microbiology

*DOI:*
10.1111/mmi.13312

**IMPORTANT NOTE:** You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

*Document Version*
Publisher's PDF, also known as Version of record

*Publication date:*
2016

*Link to publication in University of Groningen/UMCG research database*

*Citation for published version (APA):*

*Copyright*
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

*Take-down policy*
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.
Highly conserved nucleotide phosphatase essential for membrane lipid homeostasis in *Streptococcus pneumoniae*

Kirsten Kuipers,‡ Clement Gallay,‡ Václav Martinek,† Manfred Rohde,‡ Markéta Martinková,‡ Samantha L. van der Beek,† Wouter S. P. Jong,‡ Hanka Venselaar,§ Aldert Zomer,§ Hester Bootsma,§ Jan-Willem Veenimg and Marien I. de Jonge‡†

1 Laboratory of Pediatric Infectious Diseases, Department of Pediatrics, Radboud Institute for Molecular Life Sciences, Radboud university medical center, Nijmegen, The Netherlands.  
2 Molecular Genetics Group, Groningen Biomolecular Sciences and Biotechnology Institute, Centre for Synthetic Biology, University of Groningen, Groningen, The Netherlands.  
3 Department of Biochemistry, Faculty of Science, Charles University in Prague, Prague, Czech Republic.  
4 Central Facility for Microscopy, Helmholtz Centre for Infection Research, Braunschweig, Germany.  
5 Department of Molecular Cell Biology Section Molecular Microbiology, Faculty of Earth and Life Sciences, VU University, Amsterdam, The Netherlands.  
6 Center for Molecular and Biomolecular Informatics, Radboud Institute for Molecular Life Sciences, Radboud university medical center, Nijmegen, The Netherlands.  
7 Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands.  
8 Laboratory for Infectious Diseases and Screening, Centre for Infectious Disease Control, RIVM, Bilthoven, The Netherlands.

**Summary**

Proteins belonging to the DHH family, a member of the phosphoesterase superfamily, are produced by most bacterial species. While some of these proteins are well studied in *Bacillus subtilis* and *Escherichia coli*, their functions in *Streptococcus pneumoniae* remain unclear. Recently, the highly conserved DHH subfamily 1 protein PapP (SP1298) has been reported to play an important role in virulence. Here, we provide a plausible explanation for the attenuated virulence of the *papP* mutant. Recombinant PapP specifically hydrolyzed nucleotides 3'-phosphoadenosine-5'-phosphate (pAp) and 5'-phosphoadenyl-(3'–5')-adenosine (pApA). Deletion of *papP*, potentially leading to pAp/pApA accumulation, resulted in morphological defects and mislocalization of several cell division proteins. Incubation with both polar solvent and detergent led to robust killing of the *papP* mutant, indicating that membrane integrity is strongly affected. This is in line with previous studies showing that pAp inhibits the ACP synthase, an essential enzyme involved in lipid precursor production. Remarkably, partial inactivation of the lipid biosynthesis pathway, by inhibition of FabF or depletion of FabH, phenocopied the *papP* mutant. We conclude that pAp and pApA phosphatase activity of PapP is required for maintenance of membrane lipid homeostasis providing an explanation how inactivation of this protein may attenuate pneumococcal virulence.

**Introduction**

*Streptococcus pneumoniae* is a Gram-positive α-hemolytic bacterium and one of the leading causes of infection-related mortality worldwide (Walker et al., 2013). This human-specific pathogen mainly resides on the mucosal surface of the upper respiratory tract generally resulting in asymptomatic carriage (Kadioglu et al., 2008; O’Brien et al., 2009). However, *S. pneumoniae* may transit to other tissues causing a broad range of diseases varying from non-invasive infections such as otitis media and sinusitis, to more severe invasive disease, including pneumonia, meningitis and sepsis (O’Brien et al., 2009; Drijkoningen and Rohde, 2014).
Recently, the protein SP1298 of *S. pneumoniae* TIGR4 (here renamed to PapP for phosphoadenosine-5’-phosphate and 5’-phosphoadenylyl-(3’→5’)adenosine phosphatase, see results) was discovered using GAF and Tn-Seq and appears to be a highly conserved protein across the different pneumococcal serotypes (Bijlsma et al., 2007; van Opijnen and Camilli, 2012). Inactivation of this gene strongly reduced virulence at different stages of pneumococcal disease, as studied in murine models of colonization, otitis media, pneumonia, and bacteremia (Cron et al., 2011; Bai et al., 2013), suggesting a critical role for PapP in pneumococcal virulence.

PapP is annotated as DHH subfamily 1 protein as it contains the two domains DHH and DHH1. The DHH subfamily 1 is part of the superfamily of phosphoesterases (Aravind and Koonin, 1998). Within the phosphoesterases two subfamilies exist: the subfamily 2 found in eukaryotes, and the subfamily 1 found in archaea and bacteria. Although these proteins appear to be expressed by most bacteria, little is known about the function of DHH subfamily 1 proteins in *S. pneumoniae*.

PapP shares sequence homology with YtqI of *B. subtilis*, which has both oligonuclease and pAp phosphatase activity (Mechold et al., 2007). Of note, most sequence homology is shared with SMU.1297, a *Streptococcus mutans* protein that was reported as pAp phosphatase in *vitro*, but which lacks an oligonuclease activity and is required for superoxide stress tolerance (Zhang and Biswas, 2009). Recently, the PapP ortholog in strain D39 (SPD_1153) sharing 98% amino acid identity was annotated as Pde2 (PDE: phosphodiesterase) (Bai et al., 2013). They showed that PapP synergizes with SP2205 (or Pde1) in a two-step *in vitro* process, converting c-di-AMP and pApA into AMP. Based on enzymatic assays, they hypothesized a role for PapP in cell wall maintenance and signaling (Bai et al., 2013), while the conversion of pAp, as previously found for other homologous proteins, was not described. Therefore, the function of this nucleotide phosphatase remains largely enigmatic, although most studies suggest that PapP homologs convert pAp, a byproduct of the panthotenate and CoA synthesis, into AMP (McAllister et al., 2000; Mechold et al., 2007; Zhang and Biswas, 2009). More specifically, pAp is released upon generation of the acyl carrier protein (ACP), an essential precursor for the initiation of de novo fatty acid synthesis. Furthermore, it has been shown that ACP synthase activity can be inhibited by increased pAp concentrations (McAllister et al., 2000).

In this study, we demonstrate that PapP is a nucleotide phosphatase converting pAp and pApA into AMP *in vitro*. Deletion of *papP* directly influenced the cell morphology and the localization dynamics of several key cell division proteins in a temperature dependent manner. Furthermore, we discovered that the membrane integrity of the *papP* mutant was disrupted and that partial inactivation of the lipid biosynthesis process resulted in a similar *ΔpapP* phenotype, thus providing a molecular explanation for reduced *S. pneumoniae* virulence.

**Results**

**Protein conservation and structure prediction**

Protein sequence homology analyses revealed that PapP of *S. pneumoniae* TIGR4 (serotype 4) is a highly conserved phosphatase in Gram-positive bacteria, mycoplasmas and phytoplasmas. Of the 127 most conserved homologous proteins, two are well described in literature that are both 3’-phosphoadenosine-5’-phosphate (pAp) phosphatases: NmA, also known as YtqI (*Bacillus spp*.), and SMU.1297 (*Streptococcus mutans*) (Supporting Information Table S1). The latter is 68% identical to PapP (Mechold et al., 2007; Zhang and Biswas, 2009).

Homology-based structural modeling predicts PapP to be a homodimer (Fig. 1A–C). The catalytic site is formed by Asp26, Asp28, Asp82, His105 and Asp155, containing a manganese (Mn2+) ion as co-factor (Fig. 1C). No transmembrane or membrane interacting domains were identified, which suggests that PapP is a cytoplasmic protein (Fig. 1A–C). To confirm this localization, we fused a monomeric superfolder green fluorescent protein (GFP) to the C-terminal extremity of PapP. This genetic fusion was integrated at the non-essential *bgaA* locus, under the control of a zinc-inducible promoter (*papP-gfp*). As expected, the fluorescent signal was detected throughout the cell (Supporting Information Fig. S1), confirming the cytoplasmic localization of PapP.

PapP converts both nucleotide substrates pAp and pApA into AMP

To confirm that PapP is a pAp phosphatase, the protein was purified, reconstituted with Mn2+, incubated with its substrate and subjected to HPLC analysis to measure hydrolysis. Purified PapP was able to convert pAp to AMP and phosphate with a *Km* value of 580 ± 70 μM and a *Km/Kcat* of 19 ± 3 s⁻¹ M⁻¹ (Fig. 1D). The enzymatic activity increased by adding manganese, but only slightly by magnesium ions (Supporting Information Fig. S2), confirming that Mn2+ is indeed the co-factor as predicted by protein homology modeling (Fig. 1A–C). Highly efficient hydrolysis of 5’-phosphoadenylyl-(3’→5’)adenosine (pApA) by PapP was also detected with a *Km* value of 67 ± 20 μM and a *Km/Kcat* of 2100 ± 500 s⁻¹.
A similar $K_m$ value (23.96 ± 7.78 $\mu$M) was reported for the PapP ortholog from S. pneumoniae D39 named SPD_1153 or Pde2 (Bai et al., 2013). No hydrolysis of ADP, ATP, c-di-AMP and c-di-GMP was found in the presence of PapP (Supporting Information Fig. S3). This underlines the enzyme specificity for hydrolysis of 3'-phosphate groups of linear adenine-containing nucleotides, but also indicates that it is more flexible regarding the size of the substrate, as the two substrates differ significantly in their length. In conclusion, these data confirm the 3'-phosphatase activity of PapP toward pAp and pApA substrates.
S. pneumoniae deficient for papP shows aberrant morphologies

It was established that deletion of papP leads to reduction of pneumococcal virulence at many stages of disease (Cron et al., 2011). To understand the underlying mechanisms of virulence attenuation when the Pap phosphatase is absent, we investigated the cell morphology of S. pneumoniae TIGR4 strain, deficient for papP (ΔpapP). Morphology analysis by phase contrast microscopy and membrane staining with Nile red revealed that the papP mutant only forms diplococci at early exponential growth phase, while the wild type forms long chains (Fig. 2A). This is in line with the study of the PapP ortholog in S. pneumoniae D39 (serotype 2) where deletion of pde2 led to significantly shorter chains (Bai et al., 2013). Another striking observation was that the cell-poles of the papP mutant were pointier compared to the wild type cells when measuring the pole-angles of the cells (Supporting Information Fig. S4). Furthermore, cells of the papP mutant were significantly longer and had a wider cell diameter (Fig. 2B). To characterize the cell morphology in higher resolution we performed scanning electron microscopy (SEM). The micrographs confirmed that the papP deficient strain displayed a substantial shorter chain length consisting of only few cocci with sharp cell-poles (Fig. 2C). Protrusions on the cell surface appeared in all S. pneumoniae strains and could likely be capsule artifacts induced by the method (Qin et al., 2013). Complementation of papP deletion by an ectopic version of papP (ΔpapP*) reversed the diplococcus phenotype, as this strain only forms long chains and possesses a wild type cell pole angularity (Fig. 2A and Supporting Information Fig. S3). SEM pictures of this strain also confirmed that reversion of papP mutation restored the wild-type phenotype (Fig. 2C). These observations suggest that papP deficiency affects the chain formation of S. pneumoniae. In addition to morphology defects, analysis of the growth rate in liquid culture at 37°C revealed that the papP mutant demonstrates a reduced growth rate with a doubling time of 46 min (95% confidence interval 44.1 to 49.1 min) versus 34 min (95% confidence interval 35.5 to 37.4 min) for the wild type (Fig. 2D). Taken together, these results indicate that absence of PapP directly influences the cell morphology and growth of S. pneumoniae.

The polysaccharide capsule is unaffected in the absence of PapP

It is known that the polysaccharide capsule plays a crucial role in virulence of S. pneumoniae. Indeed, the non-encapsulated S. pneumoniae R6 strain (derivate from D39 strain) is non-virulent. Interestingly, strain R6 only forms diplococci instead of chains (Berg et al., 2013). Taking this into account, the phenotype observed in absence of PapP may be caused by perturbations in capsule production. To test this, we performed immunofluorescence microscopy using a serotype 4 specific capsule antibody. Interestingly, the signal illustrative for capsule presence appeared similar among wild type, mutant, and complemented strain (Fig. 3A). This suggests that despite the morphological differences, the papP mutant displayed a normal polysaccharide capsule. To confirm that loss of PapP has no effect on capsule production, bacterial lysates were assayed for the amount of capsule. A non-encapsulated mutant of TIGR4, TIGR4Δcps, was included as negative control (Fig. 3B). Strikingly, wild type, mutant and complemented strain, showed similar capsule polysaccharide levels (Fig. 3B). These data clearly indicate that the polysaccharide capsule remained intact in S. pneumoniae deficient for PapP. Hence, capsule alterations do not explain the atypical morphology of the mutant.

Effects of papP inactivation on the cell wall

To explore whether cell wall homeostasis may be influenced by inactivation of papP, qualitative and quantitative analyses were performed to probe the phosphorylcholine moieties of the pneumococcal teichoic acids. No differences were found in phosphorylcholine production between wild type, mutant, and complemented strain, as measured in a quantitative ELISA (Fig. 4A). The phosphorylcholine migration pattern as determined by Western blotting appeared identical between the three variants (Fig. 4B). These data indirectly indicate that lipoteichoic acid (LTA) and wall teichoic acid (WTA) are unaffected in the papP mutant. Additionally, no significant differences in amount of lipoproteins were measured in ΔpapP, ΔpapP*, and wild type (Supporting Information Fig. S5).

Furthermore, the effect of absence of papP on peptidoglycan was studied in a lysozyme killing assay. Notably, the papP mutant was significantly more sensitive to lysozyme, exemplified by a twofold reduced survival, as compared to wild type and the complemented mutant strain (Fig. 4C). Of note, survival of the ΔpapP strain (mean survival of 32.3%) was over 15-fold higher than a Δsp1479 strain (mean survival of 1.8%), which lacks peptidoglycan N-acetylglucosamine deacetylase A (PgdA), an enzyme directly involved in peptidoglycan cross-linking (Fig. 4C) (Davis et al., 2008). The reduced sensitivity to lysozyme as compared to the Δsp1479 mutant implies that this may not be caused by a direct effect on peptidoglycan.
**Fig. 2.** Morphological and growth defects of the \( \text{papP} \) mutant.
A. Phase contrast microscopy (left panel) and Nile red staining (right panel) of TIGR4 wild type, \( \Delta \text{papP} \) and \( \Delta \text{papP}^* \). Scale bar, 3 \( \mu \)m.
B. Cell width (\( P \)-value < 0.0001) and cell length (\( P \)-value < 0.0001) distribution of TIGR4 wild type (dark grey) and \( \Delta \text{papP} \) (green).
C. Scanning electron micrographs (SEM) of TIGR4 wild type, \( \Delta \text{papP} \) and \( \Delta \text{papP}^* \). Scale bar, 1.5 \( \mu \)m.
D. Growth curve of TIGR4 wild type (dark grey) and \( \Delta \text{papP} \) (green) at 37°C in liquid C+Y medium. SEM for TIGR4 wild type and \( \Delta \text{papP} \) are shown in clear grey and clear green respectively.
Loss of papP perturbs cell division proteins dynamics

The cell division machinery of S. pneumoniae involves many important proteins that fine-tune the cell shape (Massidda et al., 2013; Pinho et al., 2013). Deregulation of these proteins could therefore result in cell morphology defects. In order to determine whether the morphological changes of the ΔpapP mutant could be attributed to a deregulation of the division process, we inserted an ectopic copy of a N-terminal GFP fusion of the early cell division protein FtsA under control of a zinc-inducible promoter in both wild type and ΔpapP strains, resulting in strains CG16 and CG18 respectively. As expected, in the wild type cells, FtsA is recruited early at the new septum and thus localizes exclusively at mid-cell at early exponential growth phase (Fig. 5A). However, the signal in the papP mutant appears to be at mid-cell but also at the old division site (Fig. 5A). To exclude that addition of Zn²⁺ induced synthetic effects, we constructed a strain expressing a functional fusion of the early cell division protein FtsZ with the red fluorescent protein mKate2 (strains CG14 and CG22). This construct was inserted at the TIGR4 ftsZ locus, under the native promoter in order to keep the original FtsZ expression level. Fluorescence microscopy confirmed the localization of FtsZ at mid-cell in both wild type and ΔpapP (Fig. 5B). To better understand the localization dynamics of FtsZ, we performed time-lapse fluorescence microscopy at 37°C. Interestingly, while FtsZ was instantly disassembled from the old septum and reassembled at the new septum in the wild type strain (Fig. 5C, Supporting Information Movies S1 and S2), it slowly moved from the old septum to the new septum in the ΔpapP mutant (Fig. 5C, Supporting Information Movies S1 and S2). Similar results were obtained for GFP-FtsA (Supporting Information S3 Movie). To further characterize the dynamics of these two cell division proteins, the distance between FtsA or FtsZ and the closest pole of the cell was measured. When plotted with the cell length, two different clusters are clearly visible for the wild type, indicating that FtsA and FtsZ are either at the old or the new division site, or at both positions (Fig. 5D and E). However, the two distinct clusters disappeared for the mutant strain, giving way to a heterogeneous distribution (Fig. 5D and E). This indicates that FtsA and FtsZ are not only present at both the old and new septum, but also in between these two positions at one moment of the cell cycle (Fig. 5F). Taken together, these results show that inactivation of papP results in mis-localization of FtsA and FtsZ.

Fig. 3. Intact polysaccharide capsule in papP deficient S. pneumoniae.
A. Immunofluorescence staining (α-capsule) of the polysaccharide capsule of TIGR4 wild type, ΔpapP and ΔpapP* using anti-serotype four antibodies. Scale bar, 3 μm.
B. All strains were screened for capsule expression in an ELISA-based assay, with an acapsular derivative (Δcps) as negative control. Data are normalized from three independent experiments performed in duplicate. Results are illustrated at average ± SEM and compared by one-way ANOVA and Tukey’s multiple comparison test. Significance is illustrated with ***, P < 0.001; **, P < 0.01; *, P < 0.05.

Localization of cell division proteins is temperature dependent in the ΔpapP strain

To characterize the FtsZ dynamics of the papP mutant in more detail, we used time-lapse fluorescence microscopy to follow the cells on a semi-solid matrix at 30°C, a temperature known to slow down the cell cycle compared to the previous experiments performed at 37°C. Surprisingly, whereas FtsZ is well localized at mid-cell in the wild type, it appears to be completely mis-localized in the ΔpapP mutant after a few division cycles, followed by a quick death of the cells (Fig. 6A and Supporting Information Movie S4). Identical results were obtained with GFP-FtsA (Supporting Information S5 Movie). Fluorescence microscopy of cells grown at 30°C until early exponential growth phase in liquid medium confirmed the previous observations since many cells displayed abnormal morphologies and FtsZ was frequently...
mis-localized (Fig. 6B). This excludes an indirect effect of the fluorescence time-lapse microscopy technique for FtsZ mis-localization. Growth in liquid C\textsubscript{1}Y medium at 30\textdegree C also indicated a temperature sensitivity of the \textit{DpapP} strain since it entered in stationary phase at lower cell density (Fig. 6C). These data suggest that the \textit{papP} mutant is cold sensitive, especially when also harboring a FtsZ-mKate2 fusion. To further characterize the temperature sensitivity, the mutant strain was grown at even lower temperatures (25\textdegree C) and FtsZ localization was monitored. Despite a very slow growth at this temperature, FtsZ is still well localized in the wild type strain (Fig. 6D). Surprisingly, the mutant strain shows many elongated cells displaying a helical pattern of FtsZ (Fig. 6D). Thus, our data indicate that absence of the pAp phosphatase (PapP) leads to mis-localization of cell division proteins in a temperature-dependent manner.

\textit{Lack of PapP diminishes cell membrane integrity}

Many cell division proteins are directly or indirectly anchored to the cell membrane. For instance, FtsA is a membrane-associated protein that anchors FtsZ (Pichoff and Lutkenhaus, 2005). A change in membrane properties can thus impact their localization. To assess whether membrane integrity may be reduced upon \textit{papP} deletion, pneumococcal strains were screened for survival in both organic solvent and detergent killing assays. As hypothesized, the \textit{papP} mutant appeared highly susceptible for ethanol (Fig. 7A) and Triton X-100 in comparison with the wild type strain and the complemented mutant (Fig. 7B). Survival in absence of \textit{papP} was reduced approximately fivefold and fourfold in ethanol (13\%) and Triton X-100 (20\%) killing assays respectively. These data clearly indicate an increased sensitivity of \textit{S. pneumoniae} deficient for \textit{papP} to both organic and solvent and detergent, which is likely caused by an unstable and more accessible membrane. This is supported by the increased sensitivity of the \textit{papP} mutant against selected antibiotics (Supporting Information Table S4).

\textit{FabF or FabH inactivation phenocopy papP deletion}

To investigate whether the phenotype of the \textit{papP} mutant is the result of fatty acid biosynthesis perturbation, we sought to partially inactivate this process. Bacterial type II fatty-acid synthesis (FASII) inhibition seems a good strategy as these enzymes are involved in essential steps of the fatty acid biosynthesis (Parsons and Rock, 2013). For instance, the proteins FabH and FabF are respectively responsible for the initiation and elongation of the fatty acid chains. However, due to their essentiality, their inactivation by deleting the corresponding genes is not possible. On the other hand, it has

\textbf{Fig. 4.} The effect of PapP absence on cell wall components.

A. Cell-wall anchored choline levels of wild type, mutant (\textit{ΔpapP}) and complemented mutant strains (\textit{ΔpapP}) as measured by an ELISA-based assay. A higher OD\textsubscript{450} indicates a higher choline content.

B. Analysis of phosphorylcholine in the cell wall by Western blotting. The phosphorylcholine migration pattern analyzed by SDS-PAGE and Western blotting in wild type (wt), \textit{papP} mutant (\textit{ΔpapP}) and complemented (\textit{ΔpapP}) strains. The results represent phosphorylcholine migration patterns from three independent experiments (#1, #2, #3).

C. Survival of pneumococcal strains in a lysozyme killing assay, with negative control \textit{Δsp1479}. Choline expression levels show normalized data from three independent experiments performed in duplicate. Lysozyme data are combined from four independent assays. Results are provided as average ± SEM and compared by one-way ANOVA and Tukey’s multiple comparison test. Significance is illustrated with ***. \textit{P} \textless 0.001; **. \textit{P} \textless 0.01; *. \textit{P} \textless 0.05.
been reported that FabF can by inactivated by a bacteriostatic drug, platensimycin (Wang et al., 2006). To examine the effect of fatty acid synthesis inhibition by platensimycin on pneumococcal morphology, the wild type strain was grown in the presence of 0.75 μg mL⁻¹ platensimycin to early exponential growth phase and cell morphology was assessed by phase contrast microscopy. A concentration was used at which the maximum optical density of *S. pneumoniae* was reduced by two-fold (MC50) (Supporting Information Fig. S6) (Prudhomme et al., 2006). Strikingly, platensimycin treatment resulted in the formation of only diplococci (Fig. 8A) and chains of cells were no longer observed. This shows that partial inactivation of the fatty acid biosynthesis by inactivation of FabF is able to phenocopy the *papP* mutant in this regard. Next, we assessed the effect of FabH depletion on *S. pneumoniae* morphology by constructing a strain expressing an ectopic version of *fabH* under a zinc-inducible promoter and made a deletion of the native *fabH* gene in the presence of Zn²⁺ (*D fabH*). Phase contrast microscopy revealed a wild type phenotype when grown in the presence of 0.1 mM Zn²⁺ (Fig. 8B). Zinc absence is sufficient for minor expression of FabH, which is likely due to the leakage of the zinc-inducible promoter (Sorg et al., 2015). Strikingly, when grown without zinc, chain formation was absent and cells display a diplococcus phenotype (Fig. 8B). Apparently, reducing available FabH also phenocopies inactivation of *papP*. Taken together, these results strongly indicate that PapP deficiency affects the fatty acid biosynthesis resulting in the observed morphological defects.

![Fig. 5. FtsA and FtsZ dynamics are impaired in ΔpapP](image_url)

**A.** Localization of GFP-FtsA in live TIGR4 wild type and ΔpapP. The GFP fluorescent signal (left panel) is overlaid with the phase contrast image (right panel). Scale bar, 3 μm.

**B.** Localization of FtsZ-mKate2 in live TIGR4 wild type and ΔpapP. The mKate2 signal (left panel) is overlaid with the phase contrast image (right panel). Scale bar, 3 μm.

**C.** Fluorescence time-lapse microscopy of FtsZ-mKate in TIGR4 wild type and ΔpapP at 37°C. The fluorescent signal is overlaid with the phase contrast. The white arrows indicate the localization of the newly formed Z-ring. Scale bar, 0.5 μm.

**D.** Distance between GFP-FtsA and the closest pole of TIGR4 wild type and ΔpapP depending on cell size.

**E.** Distance between FtsZ-mKate2 and the closest pole of TIGR4 wild type and ΔpapP depending on cell size.

**F.** Model of TIGR4 wild type and ΔpapP cell division. Compared to the wild type situation where FtsZ (red) seems to be directly reassembled at the new division site, it seems to slowly slide from the old to the new septum in the *papP* mutant (green arrows) over the cell cycle. Cells of the *papP* mutant also appear bigger and pointer.
Discussion

The papP deletion mutant showed in a previous study decreased virulence in all stages of pneumococcal disease, which strongly indicates a critical role for papP in S. pneumoniae pathogenesis (Cron et al., 2011). Remarkably, S. pneumoniae deficient for papP showed an abnormal morphology and was substantially weaker in various killing assays compared to wild type strains and the complemented mutant. Altogether, our data indicate that loss of papP results in a cell integrity defect, rather than loss of a specific virulence factor as suggested previously (Cron et al., 2011; Bai et al., 2013).

In this study, we show that PapP is a phosphatase able to convert two adenine-nucleotide substrates, 3’-phosphoadenosine-5’-phosphate (pAp) and 5’-phospho-adenyl-(3’–>5’)-adenosine (pApA). Hydrolysis of pApA by S. pneumoniae D39 PapP ortholog (Pde2) was recently described and hypothesized to be implicated in c-di-AMP signaling and cell wall homeostasis, as pApA is a product of in vitro degradation of c-di-AMP (Corrigan et al., 2011; Bai et al., 2013). However, it is unknown in which quantities and during which other metabolic processes pApA is produced in S. pneumoniae. More importantly, its biological role remains largely unknown. A possibility is that pApA is a metabolite generated during other S. pneumoniae metabolic processes, including lipid metabolism, which may be undetectable due to a fast degradation into end products.
like AMP. This theory is supported by our observation that PapP breakdown of pApA is remarkably rapid (Fig. 1D).

In addition to pAp as substrate, Uemera and colleagues reported that Nrn degrades short RNAs, e.g. nanoRNAs. This dual preference for nanoRNA and pAp conversion has also been described for other DHH subfamily 1 proteins, including RecJ, Orn, and YtqI (Mechold et al., 2006; 2007; Wakamatsu et al., 2011), suggesting a similar activity for PapP. Whether nanoRNA degradation contributes to the phenotype of *S. pneumoniae* deficient for PapP remains speculative. Namely, Mechold and colleagues reported that RNase activity is redundant (Mechold et al., 2007), indicating that absence of a single RNase would not affect RNA metabolism. In addition, RNase activity has not been described for *S. mutans* protein SMU.1297, which shares 68% homology with PapP (Zhang and Biswas, 2009).

It is remarkable that PapP is highly conserved in Gram-positive bacteria, mycoplasmas and phytoplasmas, all containing a single membrane. In this regard, it is tempting to speculate that these single membrane-containing bacteria are more vulnerable for distortion of the lipid membrane homeostasis. Our findings demonstrate that loss of a single protein results in a weakened membrane integrity of *S. pneumoniae* (Fig. 7A and B), which makes PapP an attractive target for therapeutic interventions for Gram-positive infections.

Perturbing the membrane altered the localization dynamics of key cell division proteins FtsA and FtsZ (Figs. 5 and 6). A recent body of work has shown that cell division in *S. pneumoniae* is orchestrated by a complicated phosphorylation cascade via the eukaryotic-type serine-threonine kinase StkP (Manuse et al., 2015). It would be interesting to see what the effects of membrane perturbations, such as those caused by *papA*

![Fig. 7. Loss of PapP increases susceptibility to organic solvent and detergent.](image)

A and B. Survival of pneumococcal strains, wild type, mutant (ΔpapP), and complemented mutant strain (ΔpapP*), in an A. organic solvent ethanol and B. detergent Triton X-100 killing assay. Ethanol and Triton X-100 killing show normalized data from three independent experiments performed in duplicate. Results are provided as average ± SEM and compared by one-way ANOVA and Tukey’s multiple comparison test. Significance is illustrated with ***, P < 0.001; **, P < 0.01; *, P < 0.05.

![Fig. 8. Partial inactivation of lipid synthesis phenocopies ΔpapP morphology.](image)

A. Phase contrast microscopy (left panel) and Nile red staining (right panel) of TIGR4 wild type grown in presence of 0.75 μg/ml platensimycin. Scale bar, 3 μm.

B. Phase contrast microscopy (left panels) and Nile red staining (right panels) of ΔfabH* grown in presence of 100 μM Zn²⁺ (upper panels) or without (lower panels). Scale bar, 3 μm.
deletion, are on the signaling at the level of StkP and downstream proteins.

In conclusion, we hypothesize that in *S. pneumoniae* lacking the PAp phosphatase PapP, pAp and pApA levels will increase thereby inhibiting AcpS (McAllister *et al.*, 2000). Subsequently, decreased enzyme activity may lead to reduced precursor formation, which is required to start fatty acid synthesis, thereby hampering *de novo* lipid production (McAllister *et al.*, 2000). The resulting reduction in membrane integrity provides a plausible explanation for altered cell morphology and susceptibility and thereby reduced virulence of papP deficient *S. pneumoniae* (Cron *et al.*, 2011). Investigating newly discovered conserved pneumococcal proteins will improve our understanding of pneumococcal physiology and pathogenesis. Gaining insight into *S. pneumoniae* membrane homeostasis and virulence may reveal potential targets that will aid the development of future therapeutics and vaccines to treat and prevent pneumococcal disease.

### Experimental procedures

**Pneumococcal strains, growth conditions and transformation**

Pneumococcal strains were routinely grown at 37°C on Blood Agar (BA) or in liquid cultures of Todd Hewitt broth with 5 g L-1 yeast extract (THY). For microscopy analyses, strains were grown in C+Y medium at 37°C, 30°C or 25°C. For transformation, *S. pneumoniae* was grown in THY or C+Y medium pH = 6.8 until O.D660nm = 0.1; then 0.2 μg/mL of synthetic CSP-2 (Competence-Stimulating Peptide 2) was added and the cells were incubated at 37°C for 12 min. After DNA addition, cells were incubated at 30°C for 20 min, then diluted 10 times in THY or C+Y medium, followed by incubation for 1.5 h at 37°C. Transformants were selected on Blood agar or Columbia agar supplemented with 3% (v/v) sheep blood and the appropriate antibiotic. For induction of the P2r promoter, 100 μM ZnCl₂ was added to liquid medium or agar plates. When required, the growth medium was supplemented with 150 μg/ml spectinomycin (Sp), 500 μg/ml kanamycin (Kan), 0.5 μg/mL tetracycline (Tc) or 0.25 μg/mL erythromycin (Ery). Strains and plasmids construction are detailed in SI Methods. Strains are listed in Table 1, plasmids and primers are listed in Supporting Information Tables S2–S3 respectively.

### Bioinformatic protein conservation analyses and homology modeling

To study the conservation of SP1298 (PapP), the amino acid sequence was aligned with BLAST against the Concise Microbial Protein database with one orthologous sequence per species to avoid overrepresentation. Five hundred and fourteen proteins (e-value < 0.001) were selected and aligned using ClustalOmega (Sievers *et al.*, 2011). PapP was located in a specific clade containing 127 proteins. Proteins in this clade were subsequently coupled to species and function using the batch retrieval option in the Protein Information Resource (PIR) (Wu *et al.*, 2003) (Supporting Information Table S1).

A homology model for SP1298 was built using the experimentally solved 3D-structure of SH1221 protein from *Staphylococcus haemolyticus* as a template (PDB file 3DEV). These proteins share 43% sequence identity. A fast model algorithm with standard parameter settings in the YASARA software was used for homology modeling (Vriend, 1990).

### Protein expression and purification

The expression and purification of PapP was performed as follows. BL21-pLC1298 (Cron *et al.*, 2011) was grown at 37°C in LB medium supplemented with 0.2% glucose and 100 μg/ml ampicillin. When the culture reached an OD660 of 1.0, the incubation temperature was shifted to 18°C and growth was continued for 2 h. The culture was then diluted into fresh medium with 0.2% glucose and 100 μg/ml ampicillin to an OD660 of 0.5. A concentration of 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce the overexpression of PapP and the culture was

---

**Table 1. Streptococcus pneumoniae strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIGR4</td>
<td>Serotype 4 strain, TIGR4 wild type</td>
<td>(Tettelin <em>et al.</em>, 2001)</td>
</tr>
<tr>
<td>papP-gfp</td>
<td>TIGR4, bgaA::P₂r-papP-gfp (Tc⁺)</td>
<td>This study</td>
</tr>
<tr>
<td>CG14</td>
<td>TIGR4, ftsZ::ftsZ-mKate2 (Ery⁺)</td>
<td>This study</td>
</tr>
<tr>
<td>CG16</td>
<td>TIGR4, ftsZ::ftsZ-mKate2 (Ery⁺)</td>
<td>This study</td>
</tr>
<tr>
<td>ΔpapP</td>
<td>TIGR4, papP::spc (Sp⁺)</td>
<td>This study</td>
</tr>
<tr>
<td>Δcps</td>
<td>TIGR4, Deletion of the capsule operon</td>
<td>(Cron <em>et al.</em>, 2009)</td>
</tr>
<tr>
<td>Δsp1479</td>
<td>TIGR4, sp1479::spc (Sp⁺)</td>
<td>This study</td>
</tr>
<tr>
<td>CG22</td>
<td>TIGR4, papP::spc, ftsZ::ftsZ-mKate2 (Sp⁺, Ery⁺)</td>
<td>This study</td>
</tr>
<tr>
<td>CG18</td>
<td>TIGR4, papP::spc, bgaA::P₂r-gfp::ftsA (Sp⁺, Tc⁺)</td>
<td>This study</td>
</tr>
<tr>
<td>ΔpapP</td>
<td>TIGR4, papP::spc, IS1167::pCEP-papP (Sp⁺ Kan⁺)</td>
<td>This study</td>
</tr>
<tr>
<td>ΔfabH</td>
<td>TIGR4, fabH::ery, bgaA::P₂r-fabH (Ery⁺, Tc⁺)</td>
<td>This study</td>
</tr>
</tbody>
</table>

Sp⁺: Spectinomycin resistant; Kan⁺: Kanamycin resistant; Tc⁺: tetracycline resistant; Ery⁺: erythromycin resistant.
grown overnight at 18 °C to prevent the formation of inclusion bodies.

The cells were harvested by centrifugation, re-suspended in ice-cold PBS containing 300 mM NaCl, 5 mM Imidazole and Complete protease inhibitor cocktail (Roche), and lysed by two passages through a One Shot cell disrupter (Constant systems). Cell debris and membrane material was removed by low-speed centrifugation at 8000 × g for 20 min at 4°C and subsequent ultracentrifugation of the low-speed supernatant at 208,500 × g for 90 min at 4°C. The supernatant was loaded onto a 1 ml HiTrap TALON crude column (GE Healthcare) and PapP was eluted with a linear gradient of 5–500 mM Imidazole in PBS containing 300 mM NaCl at 125 mM Imidazole concentration. The elution peak was collected and dialyzed at 4°C with 5% glycerol.

Unbound manganese ions were removed using gel filtration on Sephadex G-50 (2.5 cm, 5 ml column) in 50 mM phosphate buffer pH 7.4 in presence of 5% glycerol.

Protein reconstitution with manganese

The concentration of purified PapP was determined spectrophotometrically at 280 nm using extinction coefficient 35 870 M⁻¹ cm⁻¹. The molar extinction coefficient was predicted by ProtParam tool (Wilkins et al., 1999). The Mn²⁺ concentration in PapP sample was determined using AAS spectrometer AAS3 (Carl Zeiss, Germany).

To ensure full saturation of the enzyme with Mn²⁺, PapP was incubated with 1:2 molar excess of MnCl₂ for 20 min at room temperature. Unbound manganese ions were removed using gel filtration on Sephadex G-50 (2.5 cm, 5 ml column) in 50 mM phosphate buffer pH 7.4 in presence of 5% glycerol.

Enzymatic activity assays

Kinetic experiments with reconstituted enzyme were conducted in 80 mM phosphate buffer (pH 7.4) containing 1 μM MnCl₂ using 0.002 and 0.1 μM enzyme for 0–10 000 μM 3′-phosphoadenosine-5′-phosphate (pAp, Santa Cruz Biotechnology, USA) and 0–300 μM 5′-phosphoadenylyl-(3′→5′)-adenosine (pApA, Biolog, USA) respectively. The reaction was stopped after 3, 6, and 9 min incubation at 25°C by immediate injection to the HPLC system. The reaction rate was determined as an increase of AMP product; different reaction stoichiometries for pApA and pAp hydrolysis were considered. The kinetic data were analyzed using non-linear Hill’s fit (considering Hill’s coefficient = 1) implemented in program Origin version 7.5 (OriginLab).

The AMP concentrations in incubation mixture were determined by ion-pair reversed-phase chromatography using the method described by Nakajima et al. (Nakajima et al., 2010). Fifteen microlitre aliquots of the incubation mixtures were injected into a monolithic C18 column (Chromolith RP-18e, 10 × 4.6 mm; Merck) and analyzed using Agilent 1200 HPLC System with detection at 260 nm. Mobile phase A contained 70 mM potassium phosphate buffer pH 6.0, with 4 mM Tetrabutylammonium hydrogensulphate (TBAS, Sigma-Aldrich, USA), as an ion-pair reagent; mobile phase B was methanol. The gradient was delivered at a flow rate of 2 ml/min according to the following program: 100% buffer A for 0.06 min; 0–50% linear gradient of B for 1.0 min; 50% B for 0.25 min; and 100% buffer A for 0.4 min. AMP (Sigma-Aldrich, USA), pAp and pApA standards were used for external calibration. Also ADP, ATP (Sigma-Aldrich, USA) and c-di-AMP and c-di-GMP (Biolog, USA) were analyzed as potential enzyme substrates using the same method.

Scanning electron microscopy

Bacteria were grown to early exponential growth phase (OD₆₀₀nm = 0.1) in C+Y medium and fixed with 2% formaldehyde. Scanning electron microscopy analyses were performed as described previously (Hammerschmidt et al., 2005). Briefly, for scanning electron microscopy (SEM) samples were dehydrated with a graded series of acetone on ice for 15 min for each step. Samples were subjected to critical-point drying with liquid CO₂ (CPD030; Balzers, Liechtenstein). The dried samples were covered with an approximately 10-nm-thick gold film by sputter coating (SCD040; Balzers Union, Liechtenstein) before examination with a field emission scanning electron microscope (Zeiss DSM 982 Gemini) using an Everhart Thornley SE detector and an in-lens detector at a 50:50 ratio at an acceleration voltage of 5 kV.

Phase contrast and epifluorescence microscopy

S. pneumoniae was grown in C+Y medium to early exponential growth phase (OD₆₀₀nm = 0.1), washed once in PBS and spotted onto a PBS-agarose slide. Microscopy pictures were acquired using a DV Elite microscope (Applied Precision) with either a sCMOS (PCO) or a CoolSNAP HQ² (Photometrics) camera, using Solid State Illumination (Applied Precision), with a 100× oil-immersion objective. Phase contrast images were acquired with 200 ms exposure time. To visualize the GFP fluorescence, a GFP filter (excitation 461/489 nm; emission 501/559 nm) was used. The mKate2 fluorescence was monitored using a mCherry filter (excitation 562/588 nm; emission 602/648 nm). The exposure time used for both GFP and mKate2 fluorescence was 600 ms. Pictures were acquired and deconvolved using Softworx (Applied Precision) and analyzed using Fiji (http://fiji.sc).

Membrane staining fluorescence microscopy

To visualize the membrane of S. pneumoniae with Nile Red, the cells were grown in C+Y medium to early exponential growth phase (OD₆₀₀nm = 0.1) and incubated with 5 μg/mL Nile red for 5 min. The cells were washed once in PBS and spotted onto a PBS-agarose slide. Fluorescence acquisition of Nile red was performed as described above using a TRITC filter (excitation 528/556 nm; emission 571/616 nm) with an exposure time of 300 ms. Pictures were acquired with Softworx and analyzed using Fiji.
Time-lapse fluorescence microscopy

*S. pneumoniae* was grown in C+Y medium to late log phase (OD600nm = 0.3) and diluted 100 times in fresh C+Y medium supplemented (when appropriate) with 0.1 mM ZnCl2 and incubated for 45 min. Cells were washed once in fresh C+Y medium and spotted onto a polyacrylamide (10%) slide incubated with C+Y medium, if required complemented with ZnCl2. Acrylamide pieces were placed inside a Gene Frame (Thermo Fisher Scientific) and sealed with the cover glass essentially as described (de Jong *et al.*, 2011). Acquisition of fluorescence was performed on a DV Elite microscope with a sCMOS camera at 37°C or 30°C. The filters used to visualize the GFP or mKate2 fluorescence are the same as described above. Exposure time used was 500 ms with 32% excitation light. Movies were acquired and deconvolved using Softworx and analyzed using Fiji.

Immunofluorescence microscopy

For fluorescence analysis of the polysaccharide capsule of *S. pneumoniae*, the strains were grown in C+Y medium at 37°C to exponential growth phase (OD600nm = 0.1), then 1:1000 diluted serum anti-serotype 4 from rabbit (Neufeld antisera, Statens Serum Institut) was added for 5 min at 4°C. Afterward, the cells were washed 3 times in pre-warmed C+Y medium and 1 μg/mL of secondary antibody anti-rabbit coupled to Alexa Fluor 555 (Invitrogen) was added for 5 min at 4°C. The cells were then washed once again in pre-warmed C+Y medium and once with PBS before being spotted onto a PBS-agarose slide. Acquisition of the fluorescent signal was performed on Nikon Ti-E microscope (Nikon) equipped with a CoolSNAP HQ2 camera, an Intensilight light source, with a 100x oil-immersion objective. To visualize the Alexa Fluor 555 fluorescence, a TRITC filter (excitation 528/556 nm; emission 571/616 nm) was used with an exposure time of 800 ms. Pictures were analyzed afterwards with Fiji.

Capsule quantification

Equal mid-log phase bacterial amounts were re-suspended in deionized water and chloroform and shaken for 5 min using TissueLyser LT (Qiagen). Subsequent centrifugation at 13,000 g for 10 min resulted in layer separation of which the aqueous layer contained polysaccharides. These samples were subjected to plates coated with anti-serotype 4 (Statens Serum institute, Neufeld Antiseri) followed by incubation with mouse anti-prevnar sera and subsequent alkaline-phosphatase-conjugated anti-mouse IgG (Sigma Aldrich). After each step the plates were washed with Phosphate Buffered Saline supplemented with 0.1% Tween-20 (PBST). Substrate solution (p-nitrophenyl phosphate in 10 mM diethanolamine and 0.5 mM magnesium chloride buffer pH 9.5) was added and absorbance was measured at 405 nm.

Quantitative analyses of cell wall phosphorylcholine

Equal amounts of mid-log phase grown pneumococci were resuspended in PBS and lysed in 0.5 gram acid-washed glass beads (150–212 μm) for 5 min at max speed using a TissueLyser LT (Qiagen). Maxisorp high binding affinity plates (Nunc) were coated with bacterial lysates in carbonate coating buffer (0.1 M carbonate/bicarbonate pH 9.6) at 4°C overnight. For choline detection, plates were incubated with mouse monoclonal TEPC-15 (Sigma) and secondary HRP-conjugated Rabbit anti-mouse antibody (DAKO). Signal was developed using 3,3’5,5’-Tetramethylbenzidine (TMB) phospho-citrate buffer and the reaction was stopped using 1.8 M sulfuric acid. Absorbance was measured at 450 nm. Detection of the choline migration pattern using Western Blot is described in the Supporting Information Materials and Methods.

Lysozyme, ethanol and Triton X-100 killing assays

Mid-log phase bacterial pellets were re-suspended in 1 mg/mL lysozyme (Merck), 25% ethanol (Merck), or 0.025% Triton X-100 (Sigma). Incubation with lysozyme was performed at 37°C for 30 min and incubation with ethanol or Triton X-100 was done at room temperature for 10 min. At indicated time points (0, 10 or 30 min), samples were 10-fold serially diluted in PBS and spotted onto BA plates that were incubated at 37°C overnight. The following day, single colonies were counted and percentage survival was calculated.

Growth curve assay

To follow the growth of *S. pneumoniae*, the strains were grown in C+Y medium at 37°C to early exponential growth phase (OD600nm = 0.1), then diluted to an O.D 600nm = 0.001. The growth was monitored in a microtiter plate reader (TECAN Infinite F200 Pro) by measuring the OD600nm every 10 min either at 37°C or 30°C. Each growth curve assay was performed in triplicate.

Statistical tests

All statistical analyses were performed using GraphPad Prism version 5.0 (Graphpad Software). Quantification of capsule and cell wall phosphorylcholine, and the killing assays were repeated in at least three independent experiments all performed in duplicate. Data were normalized to wild type assayed in the control condition (PBS), and illustrated as mean ± standard error of the mean (SEM). Groups were compared with one-way analysis of variance (ANOVA) and Tukey’s multiple comparison post-test. A P-value of ≤0.05 was considered statistically significant.

Acknowledgements

K.K. and M.d.J. were supported by Agentschap NL [Pneumovac, nr. OM111009]. Work in the Veening lab is supported by the EMBO Young Investigator Program, a VIDI fellowship (864.12.001) from the Netherlands Organisation for Scientific Research, Earth and Life Sciences (NWO-ALW) and ERC starting grant 337399-PneumoCell. V.M. and M.M. were supported by Charles University in Prague (UNCE 204025/2012). The authors thank Dr. Jakub Hraníček from the Charles
University in Prague, for assistance with determination of Mn\(^{2+}\) concentration in the PapP sample using AAS analysis, Prof. Sven Hammerschmidt and Dr. Franziska Voß for providing the \(\varepsilon\)-enolase \(\varepsilon\)-MetQ, \(\varepsilon\)-PpmA, \(\varepsilon\)-PsaA, and \(\varepsilon\)-StrA antibodies, and Dr. Jeroen Langereis for his helpful input. The authors have no conflict of interest to declare.

References


© 2015 John Wiley & Sons Ltd, Molecular Microbiology, 101, 12–26


Supporting information

Additional supporting information may be found in the online version of this article at the publisher’s web-site.