# Self-assembling shell proteins PduA and PduJ have essential and redundant roles in bacterial microcompartment assembly

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## Abstract

Protein self-assembly is a common and essential biological phenomenon, and bacterial microcompartments present a promising model system to study this process. Bacterial microcompartments are large, protein-based organelles which natively carry out processes important for carbon fixation in cyanobacteria and the survival of enteric bacteria. These structures are increasingly popular with biological engineers due to their potential utility as nanobioreactors or drug delivery vehicles. However, the limited understanding of the assembly mechanism of these bacterial microcompartments hinders efforts to repurpose them for non-native functions. Here, we comprehensively investigate proteins involved in the assembly of the 1,2-propanediol utilization bacterial microcompartment from *Salmonella enterica* serovar Typhimurium LT2, one of the most widely studied microcompartment systems. We first demonstrate that two shell proteins, PduA and PduJ, have a high propensity for self-assembly upon overexpression, and we provide a novel method for self-assembly quantification. Using genomic knock-outs and knock-ins, we systematically show that these two proteins play an essential and redundant role in bacterial microcompartment assembly that cannot be compensated by other shell proteins. At least one of the two proteins PduA and PduJ must be present for the bacterial microcompartment shell to assemble. We also demonstrate that assembly-deficient variants of these proteins are unable to rescue microcompartment formation, highlighting the importance of this assembly property. Our work provides insight into the assembly mechanism of these bacterial organelles and will aid downstream engineering efforts.

**Abbreviations:** Bacterial microcompartments, MCP; bacterial microcompartment-domain, BMC-domain;1,2-propanediol, 1,2-PD; 1,2-propanediol utilization, Pdu; signal sequence from PduD, ssD; signal sequence from PduP, ssP; transmission electron microscopy, TEM

**Keywords:** 1,2-propanediol utilization MCP, gene knockout, oligomerization, *Salmonella enterica* serovar Typhimurium LT2, rapid self-assembly assay

## Introduction

Proteins play integral and myriad roles in biological functions. Their study spans and interconnects a vast number of research fields, including genome editing with CRISPR and enzyme-driven bioremediation. Just as proteins tie together research fields, proteins themselves interact and work together to build intricate assemblies and execute complex functions. These connections can come in the form of small, transient interactions such as signal transduction through G-protein coupled receptors, or as massive, stable structures such as bacterial flagella, whose assembly extends outside the cell and help propel bacteria through their environment. In the latter case, protein self-assembly, the process by which copies of identical proteins organize into regular structures like bricks in a wall, plays a crucial role. In fact, protein self-assembly is a wide-spread phenomenon and is essential for key cellular structures and functions. Precise self-assembly of cytoskeletal elements [1] is critical for cell motility and structure [2], and controlled, rapid self-assembly is necessary for the propagation of numerous viruses [3,4]. While proper protein self-assembly is essential for numerous basic functions, improper or uncontrolled self-assembly can have dire consequences as well. For example, unregulated propagation of α-synuclein [5] or β-amyloid [6] fibers is associated with neurodegenerative diseases. Similarly, self-assembly of mutated hemoglobin into long fibers causes sickle cell anemia [7]. As such, both proper and improper protein self-assembly are essential topics of biological study.

Bacterial microcompartments (MCPs) are one example of protein self-assembly contributing to a cellular function. These are large (100-200 nm) protein-based organelles found in as many as two thirds of all bacterial phyla [8–10]. MCPs are especially common in enteric bacteria, including harmless microbes present in the human gut microbiome as well as

pathogens such as *Salmonella enterica* and *Escherichia coli*. These organelles encapsulate enzymes necessary to metabolize niche carbon sources found in the gut of hosts [11,12]. The encapsulated enzymes often produce a toxic intermediate, which is thought to be retained within the assembled protein shell [13,14]. Thus, MCPs protect the cell from harmful levels of toxic intermediates and facilitate safe, efficient metabolism.

One of the most well-studied MCPs is the 1,2-propanediol utilization (Pdu) MCP from *Salmonella enterica* serovar Typhimurium LT2. A single 22-gene operon encodes the MCP, which includes enzymes for 1,2-propanediol (1,2-PD) metabolism and cofactor recycling, as well as structural proteins that comprise the MCP shell [11,15–17]. While MCPs are believed to play a role in the survival and proliferation of *Salmonella*, these structures have also grown in popularity due to their potential utility in metabolic engineering [18–20]. Synthetic biologists are repurposing Pdu MCPs to encapsulate non-native, industrially relevant pathways to increase pathway flux [18]. MCPs offer benefits such as a known, modular mechanism for enzyme encapsulation and the native ability to recycle cofactors while preventing off-target interactions in the cytosol [16,17,21–24]. For researchers to fully harness these benefits and capabilities of MCPs, knowledge of MCP shell assembly is critical, but pieces are still missing to this mechanistic puzzle.

In this work, we demonstrate that two of the Pdu shell proteins, PduA and PduJ, have a unique, intrinsic propensity for self-assembly into long tubes upon overexpression. This builds on work suggesting that some MCP shell proteins can form various aberrant structures when overexpressed [25–27]. Furthermore, we present a microscopy-based method for the rapid quantification of this self-assembly property that does not require protein purification. We hypothesized that the tendency to self-assemble make PduA and PduJ important for MCP assembly as a whole. To test this hypothesis, we performed genomic knockouts of *pduA* and *pduJ* and demonstrate that these two genes are essential and redundant for MCP assembly. We further show that the loss of assembly cannot be overcome by other Pdu shell proteins, and

that assembly-deficient mutants of these proteins do not recover MCP assembly. Findings from this study also provide a new, non-assembled MCP control for studies investigating or modifying the MCP shell or evaluating the encapsulation of heterologous pathways. Overall, this work determined essential components of MCP shell formation and provides additional insight for engineering MCPs.

## Results

*PduA and PduJ self-assemble into tubes when overexpressed*

The *pdu* operon in *Salmonella enterica* serovar Typhimurium LT2 contains open reading frames for eight experimentally-verified shell proteins (*pduA, -B, -B', -J, -K, -N, -T,* and *-U*) [11,15,28]. Some of these shell proteins self-assemble into large, multimeric structures when overexpressed [25–27,29–31]. Indeed, when we overexpress the Pdu shell proteins PduA and PduJ in *E. coli* BL21 cells, we observe that they self-assemble into long, hollow tubes (Figure 1A). These tubes are observable in the cytoplasm when cells overexpressing PduA or PduJ are sectioned, stained, and imaged (Figure 1A). PduA and PduJ tubes are also visible via transmission electron microscopy (TEM) after purification from cells using differential centrifugation (Figure 1A).

Cells overexpressing PduA or PduJ exhibited a severe cell division defect upon induction and expression (Figure 1B). Cells with this defect appeared primarily as long chains of contiguous cells, but occasionally appeared as highly-elongated single cells (Figure 1B). Cells induced for PduA or PduJ expression were significantly longer than uninduced cells (*i.e.,* no arabinose added to cultures containing *pduA* or *pduJ* open reading frames in arabinose-inducible vectors) ($p < 0.0001$) (Figure 1C). On average, induced cells were approximately 13-21 µm long while uninduced cells were approximately 2 µm long ($p < 0.0001$) (Figure 1C). Over 90% of cells expressing PduA or PduJ existed in chains of three or more cells or single cells greater than 10 µm long, while fewer than 5% of uninduced cells were linked or elongated (p <

0.001) (Figure 1D). This method for assembly quantification via counting linked or elongated cells was more rapid than measuring cell length using an imaging software, and the two methods correlated well ($R^2 = 0.94$) (Figure S1A).

To determine whether the cell division defect is unique to PduA and PduJ, we overexpressed the other Pdu shell proteins (Figure 1B-D). Fewer than 6% of all uninduced cells or cells induced for PduB, -K, -N, -T, or -U expression were linked or elongated, significantly fewer than cells expressing PduA or PduJ ($p < 0.001$) (Figure 1D). Cells expressing shell proteins other than PduA or PduJ were also significantly shorter on average ($p < 0.0001$) (Figure 1C). We confirmed expression of all FLAG-tagged shell proteins by western blot (Figure S1B), which demonstrated that the observed differences in assembly were not due to lack of expression. Together, in support of previous studies, these results suggest that PduA and PduJ but not the other Pdu shell proteins self-assemble into extraordinarily long tubes that cause a severe cell-division defect when overexpressed in *E. coli* [25–27,29–32]. While previous works demonstrated that other shell proteins can form smaller-scale aberrant structures upon overexpression [25–27], our results emphasize major differences in the nature and degree of self-assembled PduA and PduJ structures relative to those formed by other shell proteins. This finding led us to hypothesize that PduA- and PduJ-specific self-assembly into tubes is an important property of these proteins, which is linked to overall MCP assembly.

*PduA and PduJ are essential and redundant for microcompartment assembly.*

We hypothesized that PduA and PduJ are essential for MCP assembly, but previous studies suggest that individual knockouts of either *pduA* or *pduJ* from the *pdu* operon still form MCPs [33,34]. Due to the structural and sequence similarity of these two proteins (Figure S2), we further theorized that PduA and PduJ may play redundant roles in MCP assembly and compensatory effects mask the importance of these proteins in single knockouts. To test these ideas, we created a double knockout strain of LT2 that contained neither *pduA* nor *pduJ* (ΔA ΔJ) (Figure 2A). We were unable to purify MCPs from this strain, as indicated by the absence of the

standard wild-type (WT) MCP banding pattern observed via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 2B). In contrast, the banding pattern in the single *pduA* (ΔA) and *pduJ* (ΔJ) knockouts is similar to that of the WT MCPs, apart from the missing PduA and PduJ bands at ~10 kDa. TEM on samples purified from the ΔA ΔJ double knockout strain showed only aggregates, which lacked the distinguished, flat, angular boundaries characteristic of WT MCPs (Figure 2C), whereas MCPs purified from the individual knockout strains (ΔA or ΔJ) appeared morphologically similar to WT MCPs (Figure 2C).

To further investigate the role of PduA and PduJ in MCP formation, we tested encapsulation of GFP in MCPs using a microscopy-based encapsulation assay. GFP was directed to MCPs by translationally fusing the protein with native signal sequences from PduD (ssD) and PduP (ssP) [21,22,35]. Strains capable of forming MCPs with encapsulated GFP display bright, fluorescent puncta in the cell cytoplasm (Figure 2C) while cells with uninduced *pdu* operon expression (*i.e.*, 1,2-PD not added to growth media) only exhibit diffuse GFP (Figure S3). Both the ΔA and ΔJ single knockout strains appear capable of forming MCPs as indicated by the presence of puncta throughout the cytoplasm (Figure 2C). In the ΔA ΔJ strain, we primarily observed polar bodies indicative of protein aggregation rather than MCP formation (Figure 2C). Indeed, the number of fluorescent puncta in the double knockout strain was on average 1 punctum per cell – significantly lower than the average of 3-6 puncta per cell observed in the WT strain or the single knockouts ($p < 0.0001$) (Figure 2D). The negative control ΔpocR strain, in which the positive regulatory protein pocR for the *pdu* operon is knocked out so MCP proteins are not expressed, displayed mostly diffuse GFP fluorescence with occasional puncta (Figure 2C). The rare occurrence of puncta in this strain is consistent with published data indicating that signal sequences decrease protein solubility and occasionally lead to aggregation [36]. Thus, the occasional puncta in this strain are likely aggregated GFP. The ΔA ΔJ strain also had significantly more puncta than the ΔpocR negative control ($p < 0.0001$) but significantly fewer than the WT or single knockouts (Figure 2C, 2D). Cumulatively,

these results imply that when MCPs do not assemble properly, induction of the *pdu* operon leads to more aggregation than induction of the tagged GFP constructs alone. This is likely due to the expression and aggregation of other *pdu* proteins, including enzymes and shell proteins, into a “proto-MCP” core [37,38]. These loose aggregates are then either engulfed by self-assembling shell proteins and form intact MCPs (as is the case for the WT, ΔA, and ΔJ strains), or they continue to aggregate and form polar bodies (as is the case for the ΔA ΔJ strain).

We also investigated the impact of shell modification on the function of MCP to metabolize 1,2-PD. The ΔA ΔJ strain had a variable growth pattern indicative of malformed MCPs when grown in media with 1,2-PD as the sole carbon source and supplemented with high (150 nM) adenosylcobalamin ($AdoB_{12}$) (Figure S4A). At high $AdoB_{12}$ concentrations, malformed shells initially allow rapid consumption of 1,2-PD since the encapsulated enzymes are more accessible by the substrate, leading to rapid growth [28,31,39]. At the same time, however, the toxic intermediate propionaldehyde quickly accumulates, leaks out into the cytoplasm, and causes DNA damage that leads to stalled growth [28,31,39,40]. The ΔA ΔJ double knockout strain outgrew the WT and ΔA strains initially, but then growth stalled for several hours before continuing, indicating an initial build-up and subsequent loss of propionaldehyde (Figure S4A). This pattern of accumulation and loss of propionaldehyde was also seen in MCPs with PduA point mutations [39,40]. Stalled growth in the ΔJ strain is consistent with previous reports [28,33], but surprisingly, the stalled growth for the ΔA ΔJ strain was shorter than the ΔJ strain. It is important to note that, in addition to shell assembly defects, differences in shell protein function could also cause a growth defect. Despite their structural similarity, PduA and PduJ are thought to have functional differences with regard to metabolite diffusion [34]. Thus, while impaired MCP formation is a plausible cause for the growth defect for the ΔA ΔJ double knockout strain, the functional difference of PduA and PduJ could play a role in the growth difference between the ΔA and ΔJ single knockout strains. Subtle changes to enzyme

encapsulation levels, substrate or cofactor diffusion, or MCP size could also play a role in cell growth and should be the subject of future investigations.

The results of these *in vivo* and *in vitro* assays suggest that knocking out *pduA* and *pduJ* together impairs MCP formation and function. While the outcomes of SDS-PAGE, TEM, and the GFP encapsulation assay all indicate that the ΔA ΔJ strain is unable to form MCPs, the results of the growth assay indicate subtle functional differences among the various knockout strains.

*Substitution of PduA with other Pdu shell proteins does not abolish MCP assembly*

As the single knockouts of *pduA* or *pduJ* did not prevent MCP formation, we next investigated whether other Pdu shell proteins could be substituted at the *pduA* locus without impacting MCP formation. To assess the effect of shell protein substitution, we replaced the *pduA* gene in the *pdu* operon with *pduK, -N, -U, -T,* or *-J* gene (ΔA::X*)* using the method of recombineering by Court (Figure 3A, Table 1) [41]. We also created a strain with *pduA* at the *pduJ* locus (ΔJ::A) to evaluate the effect of increasing the amount of *pduA* coded on the operon (Figure 3A, Table 1). The ability to manipulate shell expression and composition will be a valuable tool in the future efforts to re-engineer MCPs for metabolic engineering.

First, we tested whether MCPs could be purified from the modified strains. We successfully purified MCPs from each of the variants and their banding patterns on the Coomassie-stained SDS-PAGE gel were similar to WT MCPs except for the expected missing band for the substituted PduA or PduJ at ~10 kDa (Figure 3B). Interestingly, we did not see an obvious increase in the intensity of the band corresponding to the shell protein that was duplicated at the *pduA* locus (Figure 3B). It should be noted that due to the limited number of vertices possessed by an MCP shell, the concentration of the pentameric cap PduN is typically too low to visualize on an SDS-PAGE gel [28]. TEM of the purified variant MCPs showed polyhedral structures with angular edges, as we typically observe with a WT Pdu MCP (Figure 3C).

To determine whether the shell gene substitutions impact the native pathway performance, we evaluated their growth in media with 1,2-PD and 150 nM $AdoB_{12}$, as we did for the single and double knockouts of *pduA* and *pduJ*. All the ΔA::X strains except for the ΔA::U strain grew similarly (Figure S4B). The ΔA::U strain had a longer initial lag than the other strains and the greatest increase in doubling time ($t_d$) compared to the WT (Table S1) ($p < 0.0001$). We suspect the lag in the growth might be due to a transcription or translation issue, leading to delayed MCP formation, as we also observed a longer initial lag when we grew the strain with a limiting (20 nM) level of $AdoB_{12}$ (Figure S4B insert). In media with limiting $AdoB_{12}$, malformed MCPs can continue their rapid consumption of 1,2-PD without accumulating propionaldehyde to toxic levels, enabling these strains to outgrow the WT strain [39]. Instead, ΔA::U had lower cell density than WT for a large portion of the growth in the limiting $AdoB_{12}$ condition (Figure S4B insert). The lack of dependence on $AdoB_{12}$ level suggests that the ΔA::U growth defect is primarily due to reasons other than malformed MCPs.

We also evaluated the impact of Pdu shell substitution on MCP formation using the encapsulation assay with ssD-GFP (Figure 3C). As expected, we observed puncta when 1,2-PD was added to induce compartment formation (Figure 3C) and only diffuse GFP when 1,2-PD was not added (Figure S5A). The ΔA::X and ΔJ::A modified strains all displayed a higher puncta count per cell than the ΔA ΔJ strain or the negative control ΔpocR strain ($p < 0.0001$) (Figure 3D), but a lower puncta count per cell compared to the WT strain ($p < 0.003$). The reduced puncta count in the shell substitution strains could indicate a polar effect of the gene substitution that was not apparent on the SDS-PAGE gel or the growth curves, with the exception of the ΔA::U strain. The ΔA::U strain exhibited the greatest reduction in the puncta per cell count and this reduction could be the cause of the growth defect observed in the growth assay (Figure S4B). With translation or transcription issues delaying MCP formation, we hypothesized that there would be fewer MCPs available to metabolize 1,2-PD, thereby decreasing carbon flux and overall cell growth. Overall, the results indicate that substituting *pduA* with other Pdu shell

proteins still allows MCP formation but positioning other Pdu shell genes at the beginning of the operon may have some polar effects that alter growth or encapsulation, which can be further investigated in future studies.

*PduA and PduJ are not fully interchangeable and are not replaceable by other Pdu shell proteins*

After confirming that MCPs could still form with other Pdu shell proteins substituted at the *pduA* locus, we next investigated whether these shell proteins could substitute for PduA and rescue MCP formation in the ΔA ΔJ double knockout strain. To this end, we knocked out the *pduJ* gene in each of the ΔA::X strains (ΔA::X ΔJ*)* to create the double knockout substitution strains (Figure 4A). We also created a ΔA ΔJ::A strain to determine whether PduA and PduJ are fully interchangeable with one another (Figure 4A, Table 1).

Substitutions of other Pdu shell proteins at the *pduA* locus could not rescue MCP formation in the absence of both PduA and PduJ. We purified and imaged MCPs and conducted the encapsulation assay on the double knockout substitutions, as we did for the single knockout substitutions. Only MCPs purified from the ΔA::J ΔJ and ΔA ΔJ::A strains showed the banding pattern similar to WT MCPs on the SDS-PAGE gel (Figure 4B). The SDS-PAGE banding pattern for MCPs purified from the rest of the strains resembled the pattern for the ΔA ΔJ or the ΔpocR knockout strain. Similarly, TEM showed the typical MCP morphology only for samples from the ΔA::J ΔJ and ΔA ΔJ::A strains, and aggregates for samples from the rest of the modified strains (Figure 4C).

The results of the encapsulation assay bolster the analysis of the purified MCPs. Only the ΔA::J ΔJ and ΔA ΔJ::A variants gave rise to puncta counts higher than the ΔA ΔJ or ΔpocR strains in the samples induced for MCP formation ($p < 0.0001$) (Figure 4D). All strains displayed only diffuse GFP when they were not induced for MCP formation (Figure S5B). We counted more puncta per cell in the ΔA ΔJ::A variant than the ΔA::J ΔJ strain (Figure 4D). This was in contrast to the single substitution variants, in which the ΔA::J and ΔJ::A strains showed no

significant difference in the puncta per cell count (Figure 3D). Taken together, these results confirm the prior report that PduA and PduJ are structurally redundant but are not fully interchangeable [34]. To our knowledge this is the first demonstration that at least one of these proteins is required for MCP formation and other Pdu shell proteins cannot substitute for PduA or PduJ to form MCPs in the absence of both PduA and PduJ.

*Assembly-deficient pduA and pduJ point mutants do not rescue compartment assembly*

Once we established that the presence of PduA or PduJ is essential for MCP formation and cannot be replaced by other Pdu shell proteins, we then tested the hypothesis that self-assembly into tubes is a critical property for PduA and PduJ function and is linked to MCP formation. A conserved lysine residue at position 26 and 25 of PduA and PduJ, respectively, is critical for self-assembly into higher-order structures composed of multiple hexamers [33]. These conserved lysine residues are at the outer edge of the hexamers and form hydrogen bonds with adjacent hexamers in self-assembled structures [42]. Mutating these conserved lysine residues to alanine inhibits higher-order self-assembly while maintaining a folded, individual hexameric protein complex, as demonstrated in published crystallographic studies [33,34]. Taking advantage of this property, we integrated *pduA-K26A* and *pduJ-K25A* into the *pduA* locus in the *pduJ* deletion strain (Figure 5A). These strains contain only an assembly-deficient version of PduA or PduJ, allowing us to test the hypothesis that assembly-competent PduA or PduJ is essential for MCP formation.

We first verified that PduA-K26A and PduJ-K25A do not self-assemble into tubes by overexpressing these proteins in *E. coli* BL21 cells. Cells overexpressing these point mutants did not exhibit the cell division defect indicative of self-assembled proteins observed for the WT PduA and PduJ proteins (Figure 5B). While cells overexpressing WT PduA or PduJ were elongated or in chains of three or more cells over 90% of the time, less than 3% of cells overexpressing the assembly-deficient point mutants were elongated or in such chains ($p < 0.001$) (Figure 5C). This result did not appear to be due to a lack of expression of the assembly-

deficient point mutants, as both mutants were detectable on a western blot of whole cell lysate from cells expressing the proteins (Figure 5D). Our results support previous findings by Pang and Frank *et al.,* which qualitatively demonstrated that mutating the conserved lysine disrupted PduA self-assembly into aberrant structures [29].

These well-characterized PduA-K26A and PduJ-K25A assembly-deficient mutants have only been evaluated in the native system when the other self-assembling protein (WT PduJ or PduA, respectively) is still present [33], potentially confounding results. Therefore, we tested the ability of these assembly-deficient mutants to rescue MCP formation in the absence of WT PduA or PduJ. Comparison of these strains allows us to test the hypothesis that only assembly-competent versions of PduA or PduJ enable MCP formation. MCPs were purified from strains with *pduA-K26A* and *pduJ-K25A* at the *pduA* locus and compared to MCPs purified from strains with WT *pduA* and *pduJ* at the same locus (Figure 5A). These four strains also had *pduJ* knocked out, since our results with ΔA::J ΔJ indicate that PduJ can compensate for the loss of a functional protein expressed from the *pduA* locus (Figure 4). On SDS-PAGE gels, only strains containing WT *pduA* (ΔJ) or *pduJ* (ΔA::J ΔJ) at the *pduA* locus displayed the expected banding pattern of assembled MCPs (Figure 6A). By contrast, strains containing either *pduA-K26A* or *pduJ-K25A* displayed a banding pattern similar to the ΔA ΔJ strain or the ΔpocR knockout strain (Figure 6A), indicative of protein aggregation. TEM confirmed that strains containing WT *pduA* or *pduJ* produced MCPs with WT morphology, while strains containing the assembly-deficient point mutants yielded mostly protein aggregates (Figure 6B).

We further characterized MCP formation in these strains using the GFP encapsulation assay with ssD-GFP as described above. As expected, strains with WT *pduA* or *pduJ* at the *pduA* locus contained numerous bright fluorescent puncta throughout their cytoplasm when MCP expression was induced, indicative of proper MCP formation and GFP encapsulation (Figure 6B). Conversely, strains containing the assembly-deficient point mutants at the *pduA* locus displayed mostly polar bodies, similar to the ΔA ΔJ double knockout strain and indicative

of protein aggregation rather than MCP formation (Figure 6B). Strains expressing PduA-K26A or PduJ-K25A showed significantly fewer puncta per cell ($p < 0.0001$) compared to strains expressing WT PduA or PduJ, implying that the loss of self-assembly indeed inhibits the function of these proteins in MCP assembly (Figure 6C). As with other strains in this work, only diffuse ssD-GFP fluorescence was observed when MCP expression was not induced (Figure S5C). These results support our hypothesis that self-assembly is an important property for the native function of PduA and PduJ and is associated with overall MCP assembly, building on reports by Sinha *et al.* and Pang and Frank *et al.* [29,33].

*PduA and PduJ homologs do not rescue microcompartment assembly*

Self-assembly upon overexpression is a unique property of PduA and PduJ within the *pdu* operon. However, PduA homologs from other microcompartment systems can also self-assemble. For example, EutM from the ethanolamine utilization (Eut) microcompartment self-assembles into long tubes, similar to PduA [43,44]. Therefore, we hypothesized that PduA homologs might be able to rescue MCP formation, so we integrated the PduA homologs *eutM* and *csoS1A* (from a carboxysome operon in *Halothiobacillus neapolitanus*) into the *pduA* locus. These two proteins contain bacterial microcompartment domains (BMC-domains) and are structurally similar to PduA and PduJ, with small differences (Figure 7A).

The SDS-PAGE banding pattern of MCPs purified from strains containing *eutM* or *csoS1A* at the *pduA* locus and lacking *pduJ* appeared similar to that of the ΔA ΔJ strain, indicative of poorly-formed MCPs or protein aggregation (Figure 7B). Indeed, TEM of purified MCPs from these strains revealed primarily aggregated proteins (Figure 7C). The GFP encapsulation assay further confirmed these results. The ΔA::eutM ΔJ and ΔA::csoS1A ΔJ strains contained mostly polar bodies (Figure 7C), similar to the ΔA ΔJ double knockout strain, and the substituted strains had significantly fewer puncta per cell than the WT strain ($p < 0.0001$) (Figure 7D). Strains in which MCPs were not induced displayed only diffuse GFP fluorescence (Figure 7C). Combined, these results imply that Pdu MCP formation relies

specifically on PduA or PduJ, as substitution of other BMC-domain proteins was unable to produce intact MCPs. We suspect that EutM and CsoS1A are capable of self-assembly, but they might be unable to interact properly with other members of the Pdu MCP shell or enzymatic core, impairing their ability to rescue MCP function. This would support findings that EutM can be incorporated into the Pdu MCP shell, but is perhaps different enough that it cannot drive Pdu MCP assembly on its own, without the presence of PduA or PduJ [40].

## Discussion

MCPs are intriguing prokaryotic organelles that are implicated in the infectivity of certain human pathogens [45–51]. MCPs also offer a unique opportunity to address shortcomings in metabolic engineering, while providing a model system to study protein self-assembly. This incredible breadth of scope, ranging from basic understanding of protein science to infectious diseases, necessitates the development of proper controls. In this work, we uncovered an essential part of the MCP shell assembly mechanism and in the process also developed a new control for Pdu MCP shell assembly. Our findings have the potential to impact how MCPs are studied in the future.

To thoroughly and quantitatively investigate the self-assembly of two Pdu MCP shell proteins, PduA and PduJ, we developed a novel light microscopy-based method to rapidly quantify the self-assembly property without protein purification. While runaway self-assembly of PduA into tubes has been demonstrated previously [29], to our knowledge this work is the first quantitative assessment of native Pdu shell protein self-assembly in this system. Lee *et al.* used tomography on an engineered PduA homolog from *Citrobacter freundii* to assess self-assembly, but this method requires extensive sample preparation and computational analysis, and likely underestimates the extraordinary length of these structures due to the size limitations of ultra-thin sections [30]. Our light microscopy-based method enables quantification of changes to PduA or PduJ self-assembly into extended tubes without the need for purification of the protein

assemblies or modifying the native *pdu* operon. Visualization and quantification of self-assembly is rapid and straightforward, as cell division defects are easily observable under a light microscope and do not require specialized or complex software for analysis. This technique also has potential for utility outside of the Pdu MCP system in assessing assembly of other fibril-forming proteins without the need for protein purification.

The self-assembly property of PduA and PduJ appears to be a conserved trait among BMC-domain proteins from other systems. EutM in the Eut microcompartment system and RmmH from a mycobacterium operon both assemble into tubes upon overexpression [43,44,52]. Heterologous expression of carboxysome shell proteins also leads to fibril formation at high concentrations [53]. Thus, we hypothesized that a high propensity towards self-assembly is an essential feature for MCP assembly. Indeed, we showed that PduA and PduJ are essential and redundant for shell assembly, and that this is linked to their ability to individually self-assemble. It is possible that an “assembler” (*i.e.,* a protein that is prone to assembly into large structures at high concentrations) is necessary for all MCP systems. The existence of an “assembler” protein would allow other BMC domain-containing shell proteins to have specific functions apart from shell formation. For example, in the Pdu MCP system, PduB is important for enzyme encapsulation [54] while PduT may play a role in electron transfer across the shell [55,56]. These proteins might only need to retain an ability to interact with the “assembler” to be incorporated into the shell, instead of requiring a self-assembling nature of their own. Identification of the essentiality of a self-assembling protein will facilitate future investigations into the function of other MCP shell proteins as well as for the creation of a new minimal, engineered Pdu MCP with fewer shell proteins than currently published in literature [26].

In the process of uncovering the roles of PduA and PduJ self-assembly in MCP formation, we discovered that the ΔA ΔJ strain forms protein aggregates but not intact MCPs. The inability of other Pdu shell proteins to compensate for the ΔA ΔJ double knockout demonstrated the importance of having a shell protein with the self-assembling property. The

only strains that rescued MCP formation of the ΔA ΔJ double knockout strain were those with *pduJ* substituted at the *pduA* locus or *pduA* substituted at the *pduJ* locus. None of the other tested Pdu shell proteins, despite having a BMC-domain like PduA and PduJ, could form MCPs when substituted at the *pduA* locus. Thus, simply having a BMC-domain in the structure is insufficient to rescue MCP formation. Furthermore, duplicating and moving a copy of a shell protein gene upstream to the start of the *pdu* operon does not appear to affect MCP formation or shell protein incorporation significantly. For example, PduT and PduU shell proteins are thought to compose a smaller fraction of the Pdu shell than other shell proteins since they are encoded toward the end of the *pdu* operon [15]. However, the ΔA::X substitution of the tested shell proteins, including ΔA::T and ΔA::U, resulted in no obvious increase in the intensity of the corresponding band on the SDS-PAGE gel. A more quantitative (*e.g.*, using mass spectrometry) evaluation of the ratio of MCP proteins in the single substitution variants would be a valuable future avenue of exploration [57]. Understanding how to control shell composition by manipulating the operon will be an important tool for engineering the Pdu MCP shell for metabolic engineering efforts, particularly if altered transport of metabolites is required.

The findings in this work, and in particular the ΔA ΔJ strain as a new non-assembly control, have broad applicability and utility in future investigations of the native Pdu MCP function as well as in the development of Pdu MCPs as nanobioreactors. For example, the ΔA ΔJ strain will facilitate studies that require a control with unlimited substrate diffusion across the shell. Inclusion of a shell-less control also will allow comparison of encapsulated and non-encapsulated pathways, enabling evaluation of the benefits of encapsulating heterologous pathways.

The necessity of the self-assembly property and the redundancy of PduA and PduJ revealed in this work will likely influence how Pdu and other similar MCPs are engineered. Consideration of the shell protein self-assembly will be a necessary part of the future design efforts. The impact of chromosomal position on the encapsulation assay we saw with the ΔA

ΔJ::A variant versus the ΔA::J ΔJ strain also highlights the importance of being considerate of how and where these operons are manipulated. These results in our work, in addition to providing a useful control, provide a piece of the mechanistic puzzle for the assembly of these complex protein structures.

## Materials and Methods

*Plasmid and strain construction*

The strains used in this study are listed in Table 1. All plasmids were constructed using Golden Gate cloning (Table S2) [58]. Briefly, inserts containing BsaI cut sites at the 5' and 3' ends were amplified using PCR with primers listed in Table S3. These inserts were cloned into an arabinose-inducible, Golden Gate-compatible vector with a p15a origin of replication and a chloramphenicol selectable marker (pBAD33t). FastDigest Eco31I from Thermo Fisher Scientific and T4 DNA ligase from New England Biolabs, Inc. were used for the Golden Gate reaction mixture. The ligations were transformed into *E. coli* DH10b cells and successful cloning was confirmed via Sanger sequencing. For overexpression tests, plasmids were transformed into *E. coli* BL21 cells. Point mutants of PduA and PduJ were constructed using site-directed mutagenesis and KOD Hot Start polymerase (MilliporeSigma).

The *pdu* operon of *Salmonella enterica* serovar Typhimurium LT2 was modified using the λ Red recombineering method. Briefly, a *cat/sacB* selection cassette was first amplified using PCR to contain homologous 5' and 3' overhangs matching the target chromosomal location (Table S3). Using the recombineering method of the Court Lab [41], the selectable *cat/sacB* marker was inserted into the desired genomic location and then replaced with either a PCR-amplified gene of interest containing 5' and 3' homology to the target locus, or was knocked out, effectively removing the native gene. To reduce the potential polar effects from full deletion of a gene, the C-terminal 41 – 51 base pairs of *pduA* and *pduJ* loci were retained in

each modification. The modified loci were sequence confirmed via PCR amplification and Sanger sequencing.

*Shell protein overexpression*

Single colonies from *E.* coli BL21 cells streaked on agar plates were inoculated into 5 mL Lysogeny Broth, Miller (LB-M, Thermo Fisher Scientific) media in 24-well blocks. The blocks were sealed with Andwin Scientific AeraSeal™ film and grown overnight at 37 °C, 225 RPM for 15-16 hours. Overnight cultures were subcultured 1:100 (50 µL) into 5 mL of LB-M and grown at 37 °C, 225 RPM. Expression was induced with 0.2% (w/v, final concentration) L-(+)-arabinose 90 minutes after subculture, which typically corresponded to an $OD_{600}$ of ~0.3. Cells were grown at 37 °C, 225 RPM for a minimum of 4 hours post-induction and then imaged under the microscope (see *Phase contrast and fluorescence microscopy*). The LB-M cultures and agar plates included 34 ug/mL chloramphenicol.

*GFP encapsulation assay*

Overnight cultures of *S. enterica* LT2 strains containing pBAD33t-ssD-GFPmut2 plasmid (CMJ069), were prepared as described above into fresh 5 mL LB-M cultures in 24-well blocks and grown for 15-16 hours. The overnight cultures were then subcultured 1:500 (10 µL) into 5 mL of LB-M with 0.02% (w/v, final concentration) L-(+)-arabinose, 34 ug/mL chloramphenicol, and 0.4% (v/v, final concentration) 1,2-PD. Negative controls, where compartments were not induced, were grown without 0.4% (v/v) 1,2-PD in the growth media. Cultures were grown at 37 °C, 225 RPM for a minimum of 6 hours post-subculture and then imaged under the microscope (see *Phase contrast and fluorescence microscopy*).

*Growth assay*

Single colonies of the WT and chromosomally modified *S. enterica* LT2 strains were inoculated into 5 mL of Terrific Broth media (Dot Scientific, Inc.) without glycerol in test tubes and grown for 15-16 hours at 37 °C, 225 RPM. The overnight cultures were subcultured to $OD_{600}$ of 0.05 in 50 mL of No-Carbon E (NCE) media (29 mM potassium phosphate monobasic,

34 mM potassium phosphate dibasic, 17 mM sodium ammonium hydrogen phosphate) supplemented with 1 mM magnesium sulfate, 50 μM ferric citrate, 55 mM 1,2-PD, and 150 nM or 20 nM adenosylcobalamin (Santa Cruz Biotechnology). The cultures were grown in 250 mL Erlenmeyer flasks at 37 °C, 225 RPM. $OD_{600}$ measurements were taken on BioTek Synergy HTX multi-mode plate reader every 3 hours using 200 μL of sample in a clear, flat-bottom 96-well plate. From 12 hours and on, samples were diluted 1/5 or 1/10 in the culture media prior to measurement to remain within the linear range of the instrument. At least three biological replicates were measured for each strain. The doubling time ($t_d$) was calculated by $t_d = \log(2)/r$, where r is the slope of linear region in the plot of $\text{Log}(OD_{600})$ versus time.

*MCP expression and purification*

For MCP expression, single colony inoculations of *S. enterica* LT2 strains were prepared in 5 mL LB-M and grown for 24 hours at 30 °C, 225 RPM. These saturated overnight cultures were used at 1:1000 ratio to inoculate large-scale expression cultures (200 mL NCE media supplemented with 1 mM magnesium sulfate, 50 μM ferric citrate, 42 mM succinate, and 55 mM 1,2-PD). Expression cultures were grown in 1 L Erlenmeyer flasks at 37 °C, 225 RPM until cultures reached an $OD_{600}$ of at least 1.0 (usually 16-24 hours).

MCPs were purified via differential centrifugation as previously reported [59]. Briefly, once expression cultures reached the target $OD_{600}$, cells were pelleted at 5000 RPM, 4 °C, and lysed chemically using a Tris-buffered lysis solution (32 mM Tris, 200 mM potassium chloride, 5 mM magnesium chloride, 1.2% (v/v) 1,2-PD, 0.6% (w/v) octylthioglucoside (OTG), 2.2 mM β-mercaptoethanol, 0.8 mg/mL lysozyme (Thermo Fisher Scientific), and 0.04 U/mL DNase I (New England Biolabs, Inc.)). Cell lysate was clarified via centrifugation at 12,000 x *g*, 4 °C, for 5 minutes using a FIBER*Lite* F10BCI-6x500y rotor (Piramoon Technologies) in an Avanti JXN-30 centrifuge (Beckman Coulter). The clarified cell lysate was further centrifuged in a JS-24.38 rotor (Beckman Coulter) at 21,000 x *g*, 4 °C, for 20 minutes to pellet the microcompartments. The pelleted microcompartments were stored in a buffer of 50 mM Tris (pH 8.0), 50 mM

potassium chloride, 5 mM magnesium chloride, 1% (v/v) 1,2-PD at 4 °C. The purified MCPs were fixed and prepared for TEM within 10 days of purification. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) with bovine serum albumin as standard.

*Phase contrast and fluorescence microscopy*

Phase contrast and fluorescence microscopy was done on cells using Fisherbrand™ frosted microscope slides (Thermo Fisher Scientific Cat# 12-550-343) and 22 mm x 22 mm, #1.5 thickness cover slips (VWR Cat# 16004-302). Cells were imaged using a Nikon Eclipse Ni-U upright microscope and 100X oil immersion objective. GFP fluorescence was observed using the C-FL Endow GFP HYQ bandpass filter and collected with exposure time of 80 ms for strains with ssD-GFPmut2 strains and 100 ms for strains with ssP-GFPmut2. Digital micrographs were collected using an Andor Clara digital camera and NIS Elements Software (Nikon). All images were equally adjusted, within experiments, for brightness and contrast adjusted using ImageJ software [60]. Cell length measurements were collected using the segmented line tool in ImageJ [60]. Cell counts were collected on brightness and contrast-adjusted images. If a clear cleavage furrow was present on a contiguous cell body, the space flanking each furrow was counted as a single cell. For example, if a cell body contained three noticeable furrows (indicating an attempted cell-division event), this was counted as four linked cells. If a cell body was >10 µm in length, but no cleavage furrows were noticeable, the cell was counted as a single “linked” cell in the calculation for “fraction linked cells” (see Figure 1D, 5B).

*Ultra-thin section transmission electron microscopy*

The bacterial cell samples were pelleted and fixed in a solution of 2.5% Electron Microscopy (EM) grade glutaraldehyde and 2% paraformaldehyde in 0.1 M 1,4-piperazinediethanesulfonic acid (PIPES) buffer. The samples were post fixed with 1% osmium tetroxide and dehydrated in a graded series of ethanol before infiltration and embedment with EMBed 812 epoxy resin. The epoxy resin was then cured at 60 °C for 48 hours. Ultra-thin

sections were cut with a diamond knife using a Leica UC7 ultramicrotome at a thickness of 50 nm and collected on slotted grids coated with formvar and carbon. The ultra-thin sections were then stained with 3% uranyl acetate and Reynold's lead citrate prior to imaging. Data was acquired in a Hitachi HD2300 STEM at 200 kV utilizing phase contrast transmission mode and high angle annular dark field (HAADF).

*Transmission electron microscopy*

Samples were prepared and imaged via negative-stain TEM as described in-depth previously [61]. In brief, purified MCP samples were fixed on 400 mesh Formvar-coated copper grids (EMS Cat# FF400-Cu) using 2% (v/v) glutaraldehyde. Fixed samples were washed in deionized water and stained using 1% (w/v) uranyl acetate. Samples were dried and stored until imaging. Fixed and stained MCPs were imaged with the Hitachi HT-7700 Biological S/TEM Microscope and Galtan Orius 4k x 2.67k digital camera at the Northwestern Electron Probe Instrumentation Center (EPIC).

*SDS-PAGE and western blot*

For SDS-PAGE separation of purified MCPs, samples were normalized based on total protein concentration. For strains with low yields, the maximum amount of protein possible was loaded on the gel. Samples were boiled at 95 °C in Laemmli buffer for 5 minutes and loaded onto a 15% Tris-glycine mini gels. The gels were run at 120 V for 90 minutes and then stained with Coomassie Brilliant Blue R-250.

Western blots were performed on $OD_{600}$-normalized samples of whole cell lysate. A 1 mL sample of induced culture was pelleted and stored at -20 °C until use. Pellets were resuspended in 25 mM Tris base, 192 mM glycine, 1.1% (w/v) SDS to an $OD_{600}$ of 3.0. Samples were then mixed with 4X Laemmli buffer at 3:1 volume ratio and boiled for 5 minutes at 95 °C. Boiled samples were run on a 15% Tris-glycine gel at 130 V for 80 minutes and transferred to a PVDF membrane at 90 V for 11 minutes by wet transfer with Bio-Rad Mini Trans-Blot Cell. Membranes were blocked in TBS-T (20 mM Tris (pH 7.5), 150 mM sodium chloride, 0.05% (v/v) Tween 20))

with 5% (w/v) dry milk for 1 hour at room temperature. Blocked membranes were incubated overnight at 4 °C with a 1:6666 dilution of mouse anti-FLAG primary antibody (MilliporeSigma Cat# F3165) in TBS-T with 5% (w/v) dry milk. Membranes were washed with TBS-T and incubated in a 1:1000 dilution of HRP-conjugated goat anti-mouse secondary antibody (Invitrogen Cat# 32430) in TBS-T for two hours at room temperature. Finally, membranes were developed using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) and imaged using the Bio-Rad ChemiDoc XRS+ System.

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## Author Contributions

**NWK**: Conceptualization, methodology, validation, formal analysis, investigation, resources, writing – original draft, writing – review & editing, visualization, supervision, project administration, funding acquisition. **SPI**: Conceptualization, methodology, validation, formal analysis, investigation, resources, writing – original draft, writing – review & editing, visualization, project administration. **MSL**: Conceptualization, methodology, investigation, resources, writing-review & editing. **HWR**: Conceptualization, methodology, formal analysis,

investigation, writing – review & editing. **DTE**: Conceptualization, validation, resources, writing – original draft, writing – review & editing, supervision, project administration, funding acquisition.

## Declaration of Interests: none

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**Figure 1 – PduA and PduJ self-assemble into tubes when overexpressed.** (A) TEM of PduA and PduJ self-assembled into long tubes. Cell sections of *E. coli* cells show PduA (top left and middle) and PduJ (bottom left and middle) self-assembled into tubes when overexpressed. These are visible in both transverse (left) and longitudinal (middle) cell sections (scale bar = 200 nm). Black arrows indicate the tubes. Self-assembled PduA and PduJ tubes can also be purified using differential centrifugation and visualized via TEM (right top and bottom, respectively) (scale bar = 500 nm). (B) Cells induced (+ inducer) or uninduced (- inducer) for overexpressing Pdu shell proteins observed using phase contrast microscopy (scale bar = 5 µm). (C) Length of cells induced (+) or uninduced (-) for overexpression of Pdu shell proteins. Cells overexpressing PduA or PduJ (+ inducer) are significantly longer than cells overexpressing other Pdu shell proteins or not overexpressing shell protein (- inducer) ($p < 0.0001$, t-test). Measurements were taken from three biological replicates. (D) Fraction of linked or elongated cells in population of cells induced (+) or uninduced (-) for Pdu shell protein overexpression. A significantly higher fraction of cells overexpressing PduA or PduJ (+ inducer) are in linkages of three or more cells or longer than 10 µm ($p < 0.001$, t-test). Counts were taken from three biological replicates (>80 cells counted per strain per replicate), and error bars represent standard error of the means.

**Figure 2 – Double knockout of *pduA* and *pduJ* impairs MCP formation.** (A) Schematic representation of *pdu* operon and *pdu* gene knockouts. (B) Coomassie-stained SDS-PAGE gel of purified MCPs from different strains with Pdu MCP components labelled. “MWM” is the molecular weight marker and “Lys.” is lysozyme from the lysis buffer. (C) (Left) TEM of purified MCPs from different strains. The image of WT MCPs shows the distinct polyhedral shape and borders we expect from properly formed MCPs. Scale bar = 100 nm. (Right) Phase contrast and GFP fluorescence microscopy images of strains encapsulating either ssD-GFP or ssP-GFP. Row labels indicate the strain and column labels indicate the type of microscopy and the signal sequence appended to GFP. Scale bars = 3 µm. (D) Quantification of puncta per cell for strains

encapsulating either ssD-GFP or ssP-GFP. WT, ΔA, and ΔJ strains had significantly greater numbers of puncta per cell than the ΔA ΔJ strain, regardless of signal sequence used to tag GFP ($p < 0.0001$, t-test). Counts were collected from three biological replicates (>50 cells counted per strain per replicate). Overlaid data points represent the number of puncta for an individual cell and are distributed randomly around the whole number value (y-axis) to facilitate visualization.

**Figure 3 – MCPs assemble in single knockout of pduA or pduJ substituted with other Pdu shell proteins.** (A) Schematic representation of *pdu* operon, and *pdu* gene substitutions in *pduA* or *pduJ* single knockout strains. (B) Coomassie-stained SDS-PAGE gel of purified MCPs from single knockout substitution strains with Pdu MCP components labelled. “MWM” is the molecular weight marker and “Lys.” is lysozyme from the lysis buffer. (C) (Left) TEM of purified MCPs from single knockout substitution strains. Scale bar = 100 nm. (Right) Phase contrast and GFP fluorescence microscopy images of strains encapsulating ssD-GFP. Row labels indicate the strain and column labels indicate the type of microscopy. Scale bars = 3 µm. (D) Quantification of puncta per cell for strains encapsulating ssD-GFP. WT had significantly higher number of puncta than the substitution strains, ΔA ΔJ strain, or ΔpocR strain ($p < 0.01$, pairwise t-test with Holm’s adjustment), but ΔA::J and ΔJ::A strains showed no significant difference between them ($p = 1$, pairwise t-test with Holm’s adjustment). Counts were collected from three biological replicates (>24 cells counted per strain per replicate). Overlaid data points represent the number of puncta for an individual cell and are distributed randomly around the whole number value (y-axis) to facilitate visualization.

**Figure 4 – Only strains with pduA or pduJ form MCPs in double knockout substitution strains.** (A) Schematic representation of *pdu* operon, and *pdu* gene substitutions in ΔA ΔJ double knockout strain. (B) Coomassie-stained SDS-PAGE gel of purified MCPs from double

knockout substitution strains with Pdu MCP components labelled. “MWM” is the molecular weight marker and “Lys.” is lysozyme from the lysis buffer. (B) (Left) TEM of purified MCPs from double knockout substitution strains. Scale bar = 100 nm. (Right) Phase contrast and GFP fluorescence microscopy images of strains encapsulating ssD-GFP. Row labels indicate the strain and column labels indicate the type of microscopy. Scale bars = 3 µm. (D) Quantification of puncta per cell for strains encapsulating ssD-GFP. WT had significantly higher number of puncta than substitution strains ($p < 0.0001$, pairwise t-test with Holm’s adjustment). ΔA ΔJ::A strain also had higher puncta count than the ΔA::J ΔJ strain ($p<0.0001$, t-test). Counts were collected from three biological replicates (>28 cells counted per strain per replicate). Overlaid data points represent the number of puncta for an individual cell and are distributed randomly around the whole number value (y-axis) to facilitate visualization.

**Figure 5 – Point mutations disrupt PduA and PduJ self-assembly.** (A) Schematic representation of *pdu* operon and strain modifications. (B) Phase contrast microscopy images of cells overexpressing PduA, PduJ, and assembly-deficient point mutants. (C) Fraction of linked or elongated cells in population of cells induced (+) or uninduced (-) for overexpression of WT or assembly deficient PduA and PduJ. Cells overexpressing (+ inducer) PduA-WT or PduJ-WT have a significantly greater fraction of cells in linkages of three or more cells or longer than 6 µm ($p < 0.001$, t-test). Counts were taken from three biological replicates (>80 cells counted per strain per replicate), and error bars represent standard error of the means. (D) Western blot of whole-cell lysate from cells overexpressing FLAG-tagged PduA, PduJ, and assembly-deficient point mutants.

**Figure 6 - Assembly-deficient point mutants do not rescue MCP formation.** (A) Coomassie-stained SDS-PAGE gel of purified MCPs from different strains. “MWM” is the molecular weight marker, and “Lys.” is lysozyme. (B) (Left) TEM of purified MCPs from different

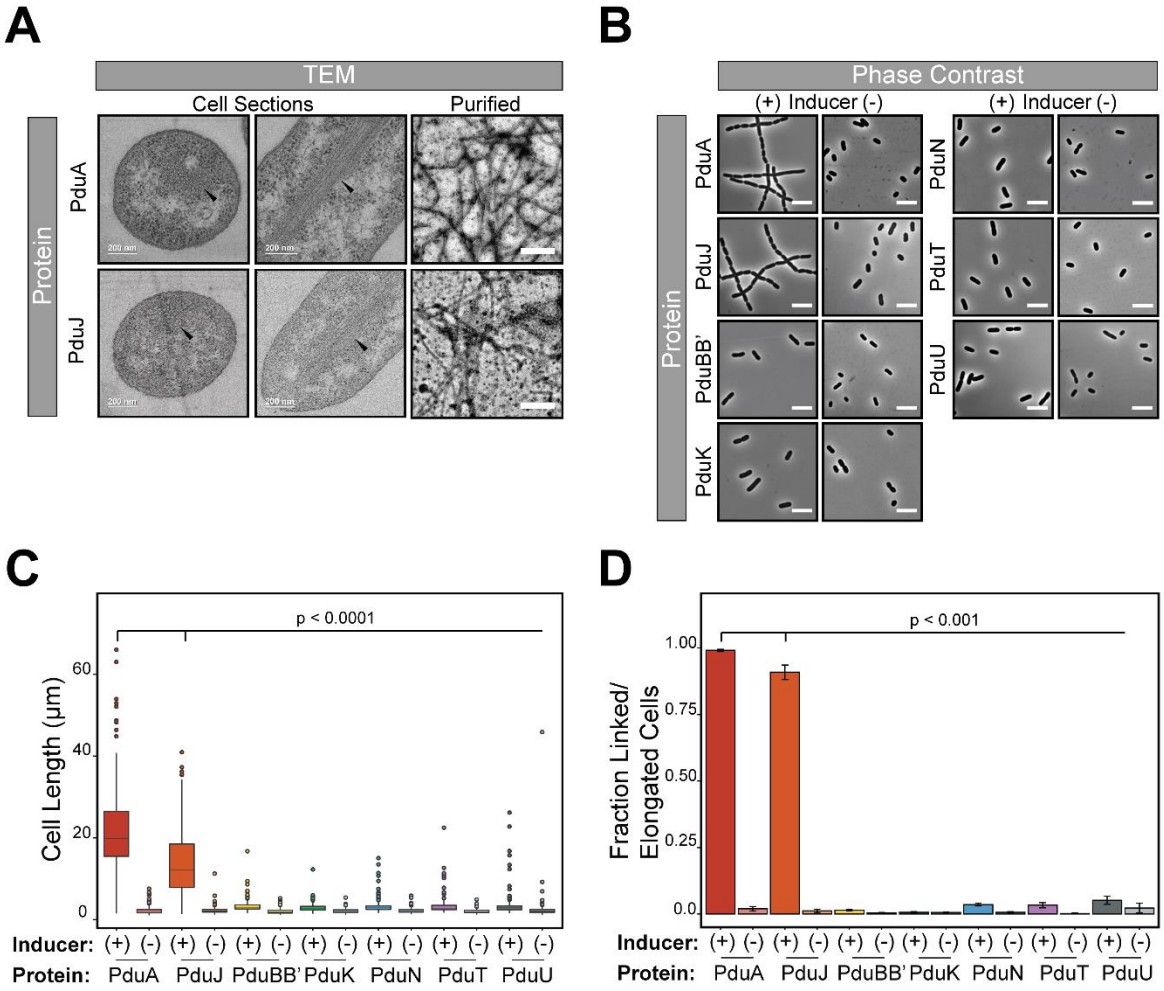
strains. Scale bars = 100 nm. (Right) Phase contrast and GFP fluorescence microscopy images of strains encapsulating ssD-GFP. Row labels indicate the strain and column labels indicate the type of microscopy. Scale bars = 3 µm. (C) Quantification of puncta per cell for strains encapsulating ssD-GFP. Strains expressing assembly-deficient shell proteins had significantly fewer puncta per cell than strains expressing either PduA-WT or PduJ-WT ($p < 0.0001$, t-test). Counts were collected from three biological replicates (>40 cells counted per strain per replicate). Overlaid data points represent the number of puncta for an individual cell and are distributed randomly around the whole number value (y-axis) to facilitate visualization.

**Figure 7 – PduA homologs do not rescue MCP formation.** (A) Structural overlay of PduA (tan, PDB ID: **3NGK**), PduJ (pink, PDB ID: **5D6V**), EutM (blue, PDB ID: **3MPY**), and CsoS1A (green, PDB ID: **2EWH**). (B) Coomassie-stained SDS-PAGE gel of purified MCPs. Strains are listed above lanes on the gel. “MWM” is the molecular weight marker and “Lys.” is lysozyme. (C) (Left) TEM of purified MCPs from different strains. Scale bars = 100 nm. (Right) Phase contrast and GFP fluorescence microscopy images of strains encapsulating ssD-GFP. Row labels indicate the strain and column labels indicate the type of microscopy. Scale bars = 3 µm. (D) Quantification of puncta per cell for strains encapsulating ssD-GFP. Strains expressing EutM or CsoS1A have significantly fewer puncta per cell than the wild-type (WT) strain ($p < 0.0001$, t-test). Counts were collected from three biological replicates (>33 cells counted per strain per replicate). Overlaid data points represent the number of puncta for an individual cell and are distributed randomly around the whole number value (y-axis) to facilitate visualization.

**Table 1.** Strains used in this study.

| Strain | Organism | Genotype |
|---|---|---|
| DTE017 | *E. coli DH10b* | Wild type |
| DTE001 | *E. coli BL21* | Wild type |
| MFSS044 | *S. enterica serovar Typhimurium LT2* | Wild type |
| TUC01 [41,62] | *E. coli W3110* | *gal490 pglΔ8 λcI857 Δ(cro-bioA) int<>cat/sacB* |
| MFSS375 | *S. enterica serovar Typhimurium LT2* | *ΔpduA* |
| NWKs081 | *S. enterica serovar Typhimurium LT2* | *ΔpduJ* |
| NWKs083 | *S. enterica serovar Typhimurium LT2* | *ΔpduA ΔpduJ* |
| SPIS051 | *S. enterica serovar Typhimurium LT2* | *ΔpocR* |
| SPIS188 | *S. enterica serovar Typhimurium LT2* | *ΔpduA::pduK* |
| MFSS409 | *S. enterica serovar Typhimurium LT2* | *ΔpduA::pduN* |
| SPIS132 | *S. enterica serovar Typhimurium LT2* | *ΔpduA::pduU* |
| MFSS411 | *S. enterica serovar Typhimurium LT2* | *ΔpduA::pduT* |
| MFSS412 | *S. enterica serovar Typhimurium LT2* | *ΔpduA::pduJ* |
| SPIS201 | *S. enterica serovar Typhimurium LT2* | *ΔpduA::EutM* |
| MFSS387 | *S. enterica serovar Typhimurium LT2* | *ΔpduA::csoS1A* |
| SPIS194 | *S. enterica serovar Typhimurium LT2* | *ΔpduJ::pduA* |
| NWKs293 | *S. enterica serovar Typhimurium LT2* | *ΔpduA::pduK ΔpduJ* |
| SPIS144 | *S. enterica serovar Typhimurium LT2* | *ΔpduA::pduN ΔpduJ* |
| SPIS145 | *S. enterica serovar Typhimurium LT2* | *ΔpduA::pduU ΔpduJ* |
| SPIS146 | *S. enterica serovar Typhimurium LT2* | *ΔpduA::pduT ΔpduJ* |
| SPIS147 | *S. enterica serovar Typhimurium LT2* | *ΔpduA::pduJ ΔpduJ* |
| SPIS189 | *S. enterica serovar Typhimurium LT2* | *ΔpduA ΔpduJ::pduA* |
| NWKs262 | *S. enterica serovar Typhimurium LT2* | *ΔpduA::pduA-K26A ΔpduJ* |
| NWKs294 | *S. enterica serovar Typhimurium LT2* | *ΔpduA::pduJ-K25A ΔpduJ* |
| NWKs253 | *S. enterica serovar Typhimurium LT2* | *ΔpduA::eutM ΔpduJ* |
| NWKs254 | *S. enterica serovar Typhimurium LT2* | *ΔpduA::csoS1A ΔpduJ* |

**Figure 1**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TEM
Cell Sections
Purified
Protein
PduA
PduJ
200 nm
B
Phase Contrast
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(+) Inducer (-)
Protein
PduA
PduJ
PduBB'
PduK
PduN
PduT
PduU
C
p < 0.0001
Cell Length (µm)
0
20
40
60
Inducer: (+) (-) (+) (-) (+) (-) (+) (-) (+) (-) (+) (-) (+) (-)
Protein: PduA PduJ PduBB' PduK PduN PduT PduU
D
p < 0.001
Fraction Linked/
Elongated Cells
0.0
0.25
0.50
0.75
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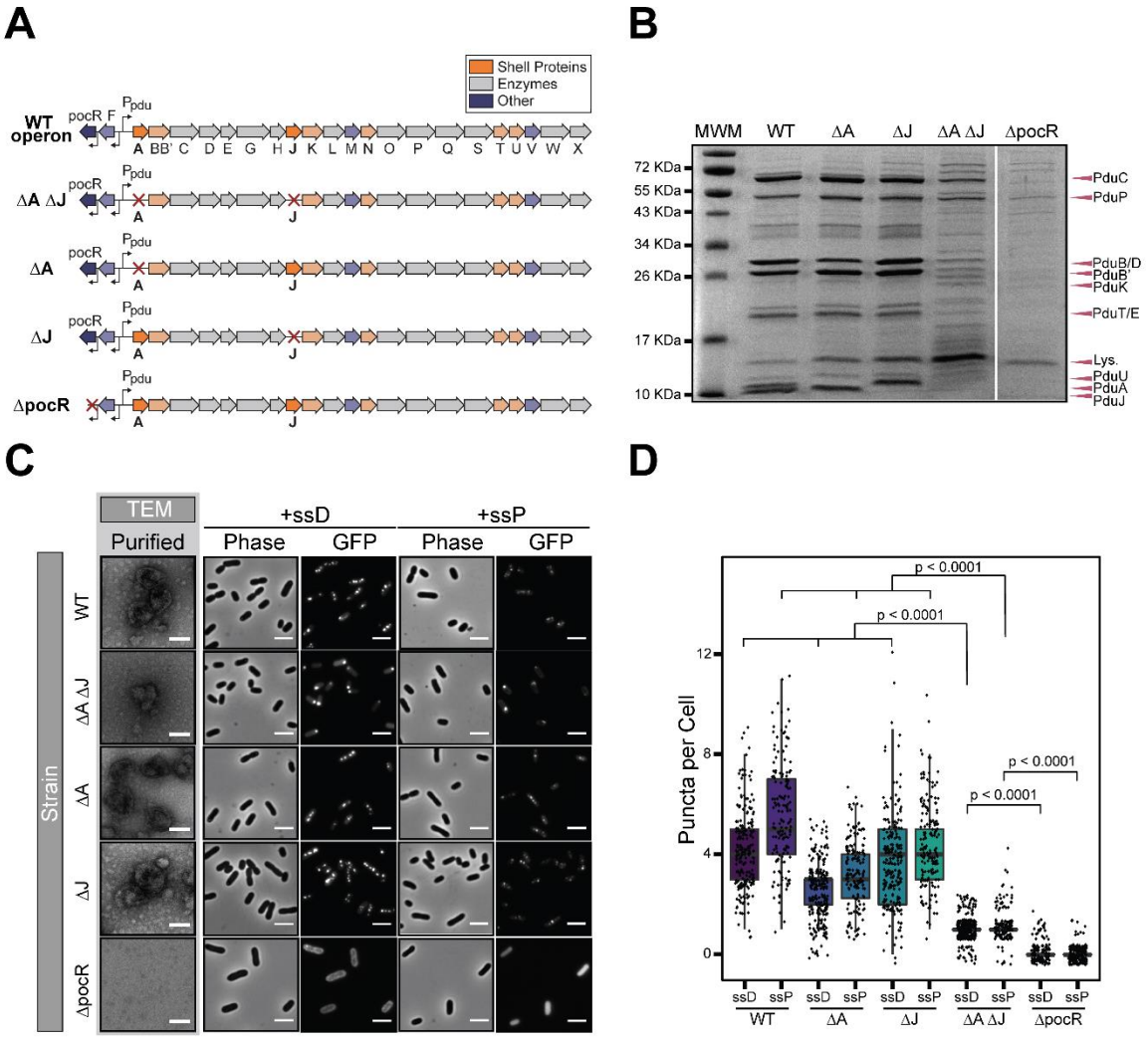
Protein: PduA PduJ PduBB' PduK PduN PduT PduU

**Figure 2**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Shell Proteins
Enzymes
Other
WT operon
pocR F
Ppdu
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ΔA ΔJ
ΔA
ΔJ
ΔpocR
B
MWM WT ΔA ΔJ ΔA ΔJ ΔpocR
72 KDa
55 KDa
43 KDa
34 KDa
26 KDa
17 KDa
10 KDa
PduC
PduP
PduB/D
PduB'
PduK
PduT/E
Lys.
PduU
PduA
PduJ
C
TEM
Purified
+ssD
+ssP
Phase
GFP
Strain
WT
ΔA ΔJ
ΔA
ΔJ
ΔpocR
D
p < 0.0001
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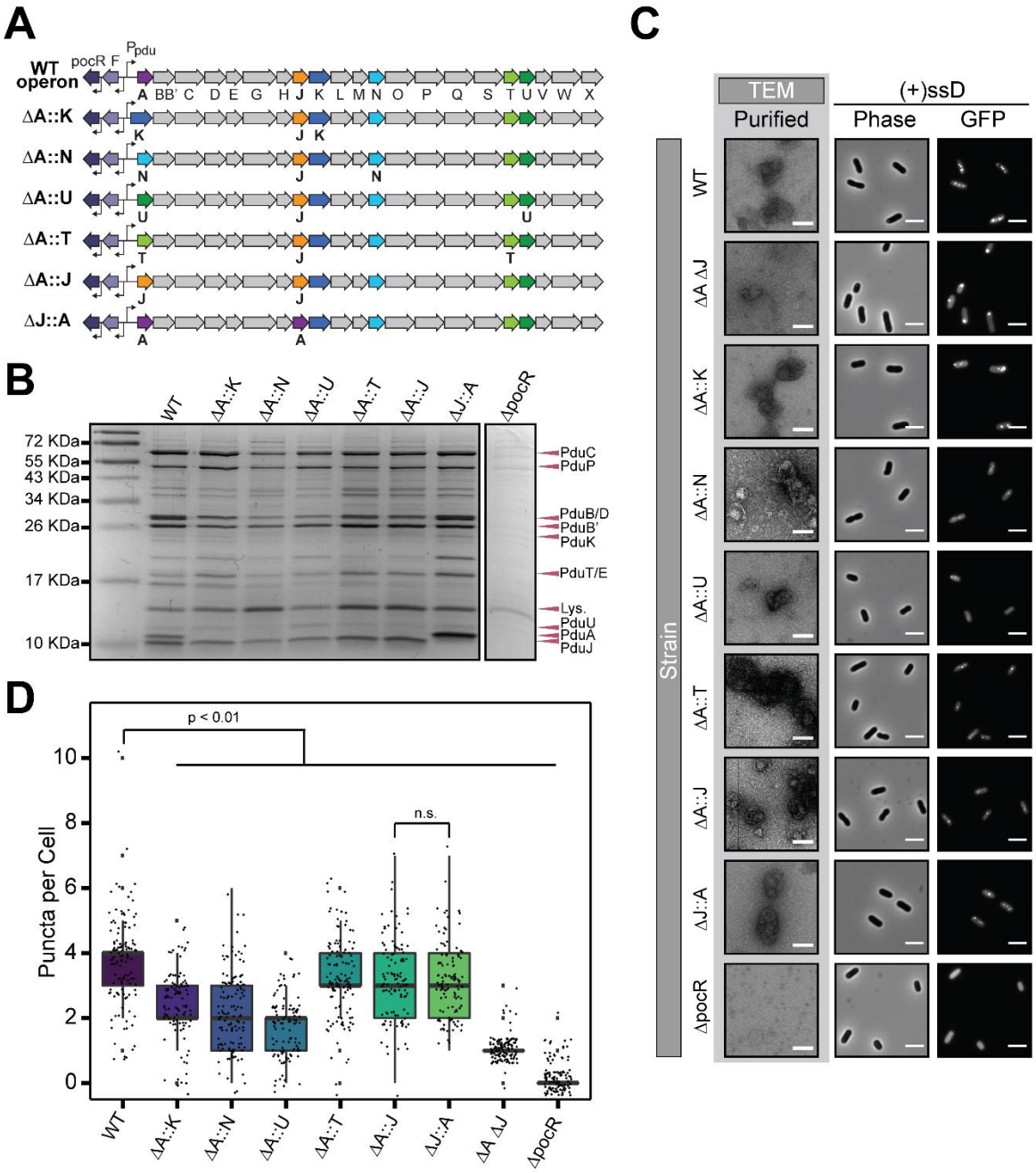
4
8
12
ssD ssP
WT
ΔA
ΔJ
ΔA ΔJ
ΔpocR

**Figure 3**


A
WT operon
pocR F
Ppdu
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ΔA::K
ΔA::N
ΔA::U
ΔA::T
ΔA::J
ΔJ::A
B
WT
ΔA::K
ΔA::N
ΔA::U
ΔA::T
ΔA::J
ΔJ::A
ΔpocR
72 KDa
55 KDa
43 KDa
34 KDa
26 KDa
17 KDa
10 KDa
PduC
PduP
PduB/D
PduB'
PduK
PduT/E
Lys.
PduU
PduA
PduJ
C
TEM
Purified
(+)ssD
Phase
GFP
Strain
WT
ΔA ΔJ
ΔA::K
ΔA::N
ΔA::U
ΔA::T
ΔA::J
ΔJ::A
ΔpocR
D
p < 0.01
n.s.
Puncta per Cell
0
2
4
6
8
10
WT
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ΔA::N
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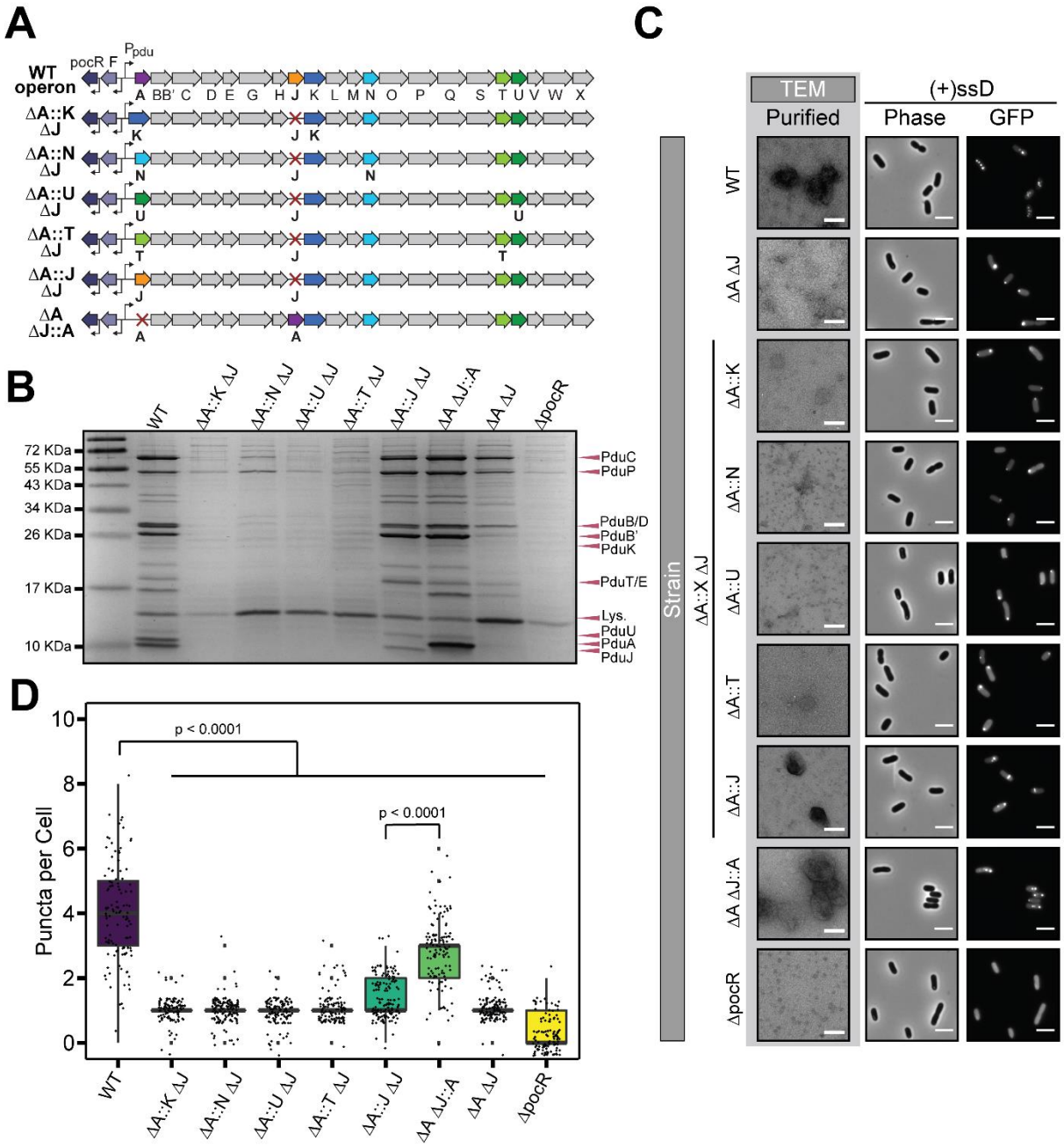
ΔA::T
ΔA::J
ΔJ::A
ΔA ΔJ
ΔpocR

**Figure 4**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WT operon
pocR F
Ppdu
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ΔA::N ΔJ
ΔA::U ΔJ
ΔA::T ΔJ
ΔA::J ΔJ
ΔA ΔJ::A
B
WT
ΔA::K ΔJ
ΔA::N ΔJ
ΔA::U ΔJ
ΔA::T ΔJ
ΔA::J ΔJ
ΔA ΔJ::A
ΔA ΔJ
ΔpocR
72 KDa
55 KDa
43 KDa
34 KDa
26 KDa
17 KDa
10 KDa
PduC
PduP
PduB/D
PduB'
PduK
PduT/E
Lys.
PduU
PduA
PduJ
C
TEM
Purified
(+)ssD
Phase
GFP
Strain
ΔA::X ΔJ
WT
ΔA ΔJ
ΔA::K
ΔA::N
ΔA::U
ΔA::T
ΔA::J
ΔA ΔJ::A
ΔpocR
D
Puncta per Cell
p < 0.0001
p < 0.0001
0
2
4
6
8
10
WT
ΔA::K ΔJ
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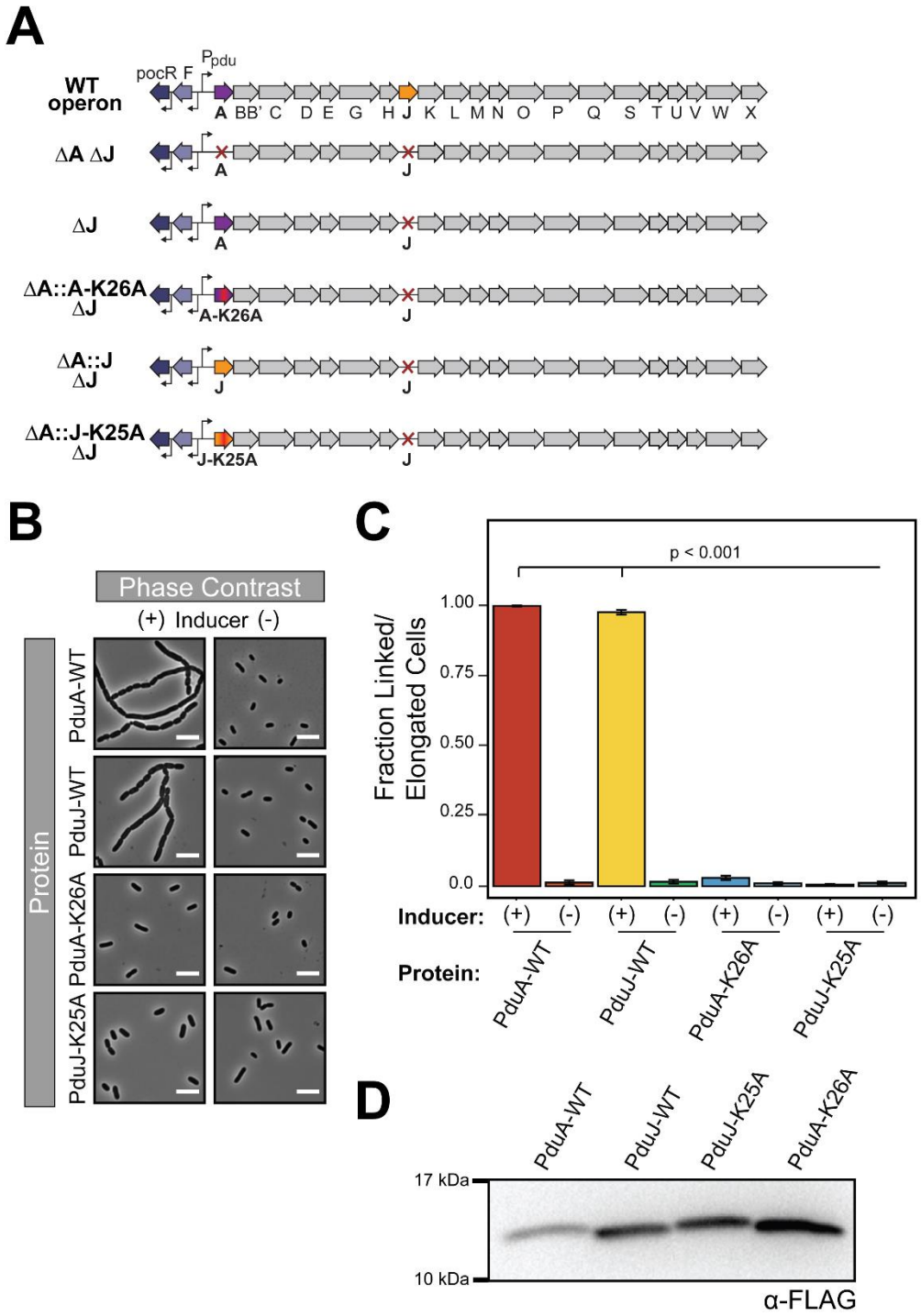
ΔA::J ΔJ
ΔA ΔJ::A
ΔA ΔJ
ΔpocR

**Figure 5**


A
WT operon
pocR F
Ppdu
A BB' C D E G H J K L M N O P Q S T U V W X
ΔA ΔJ
ΔJ
ΔA::A-K26A ΔJ
A-K26A
ΔA::J ΔJ
J
ΔA::J-K25A ΔJ
J-K25A
B
Phase Contrast
(+) Inducer (-)
Protein
PduA-WT
PduJ-WT
PduA-K26A
PduJ-K25A
C
p < 0.001
Fraction Linked/ Elongated Cells
1.00
0.75
0.50
0.25
0.0
Inducer: (+) (-) (+) (-) (+) (-) (+) (-)
Protein: PduA-WT PduJ-WT PduA-K26A PduJ-K25A
D
PduA-WT
PduJ-WT
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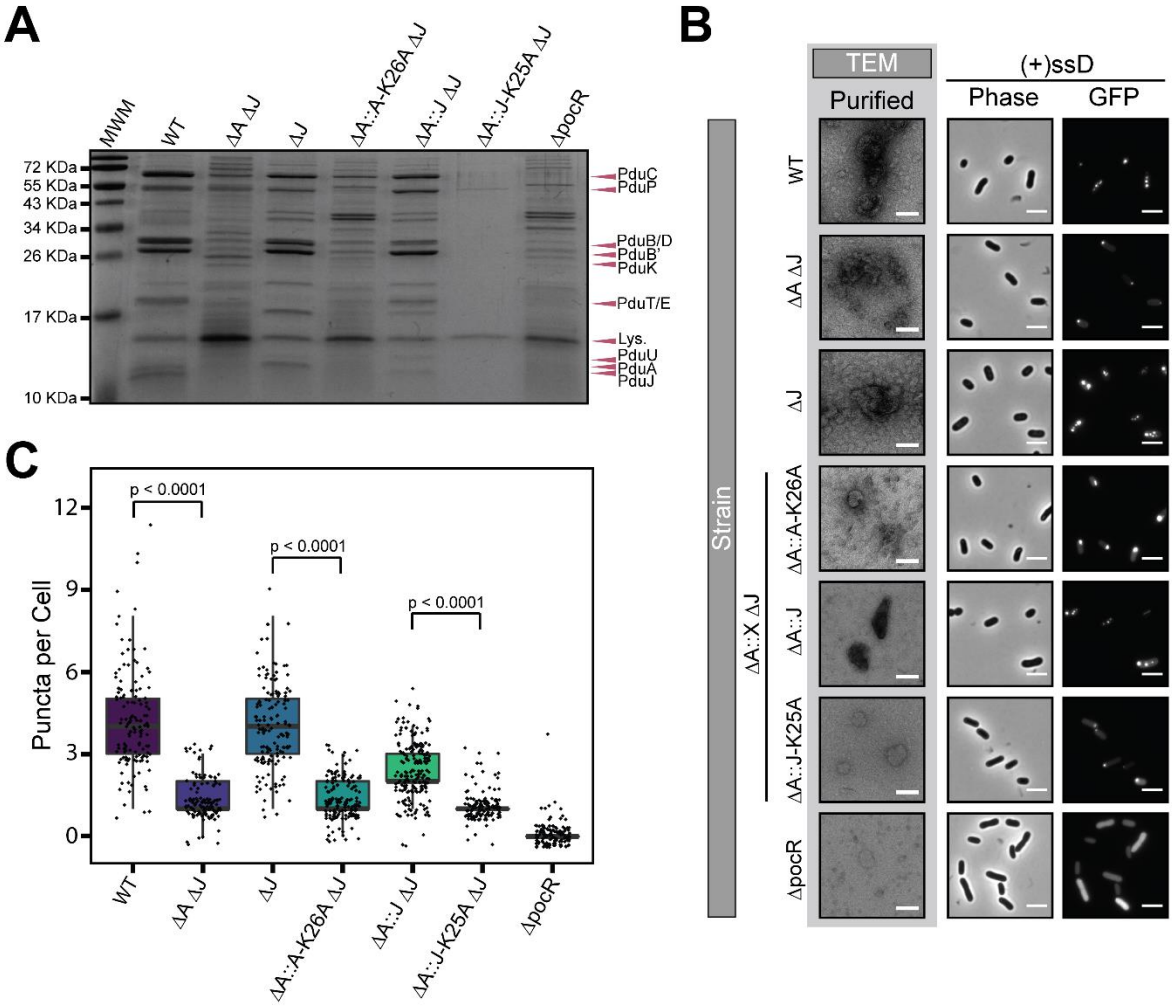
10 kDa
α-FLAG

**Figure 6**


A
MWM
WT
ΔA ΔJ
ΔJ
ΔA::A-K26A ΔJ
ΔA::J ΔJ
ΔA::J-K25A ΔJ
ΔpocR
72 KDa
55 KDa
43 KDa
34 KDa
26 KDa
17 KDa
10 KDa
PduC
PduP
PduB/D
PduB'
PduK
PduT/E
Lys.
PduU
PduA
PduJ
B
Strain
ΔA::X ΔJ
TEM
Purified
(+)ssD
Phase
GFP
WT
ΔA ΔJ
ΔJ
ΔA::A-K26A
ΔA::J
ΔA::J-K25A
ΔpocR
C
Puncta per Cell
p < 0.0001
p < 0.0001
p < 0.0001
0
3
6
9
12
WT
ΔA ΔJ
ΔJ
ΔA::A-K26A ΔJ
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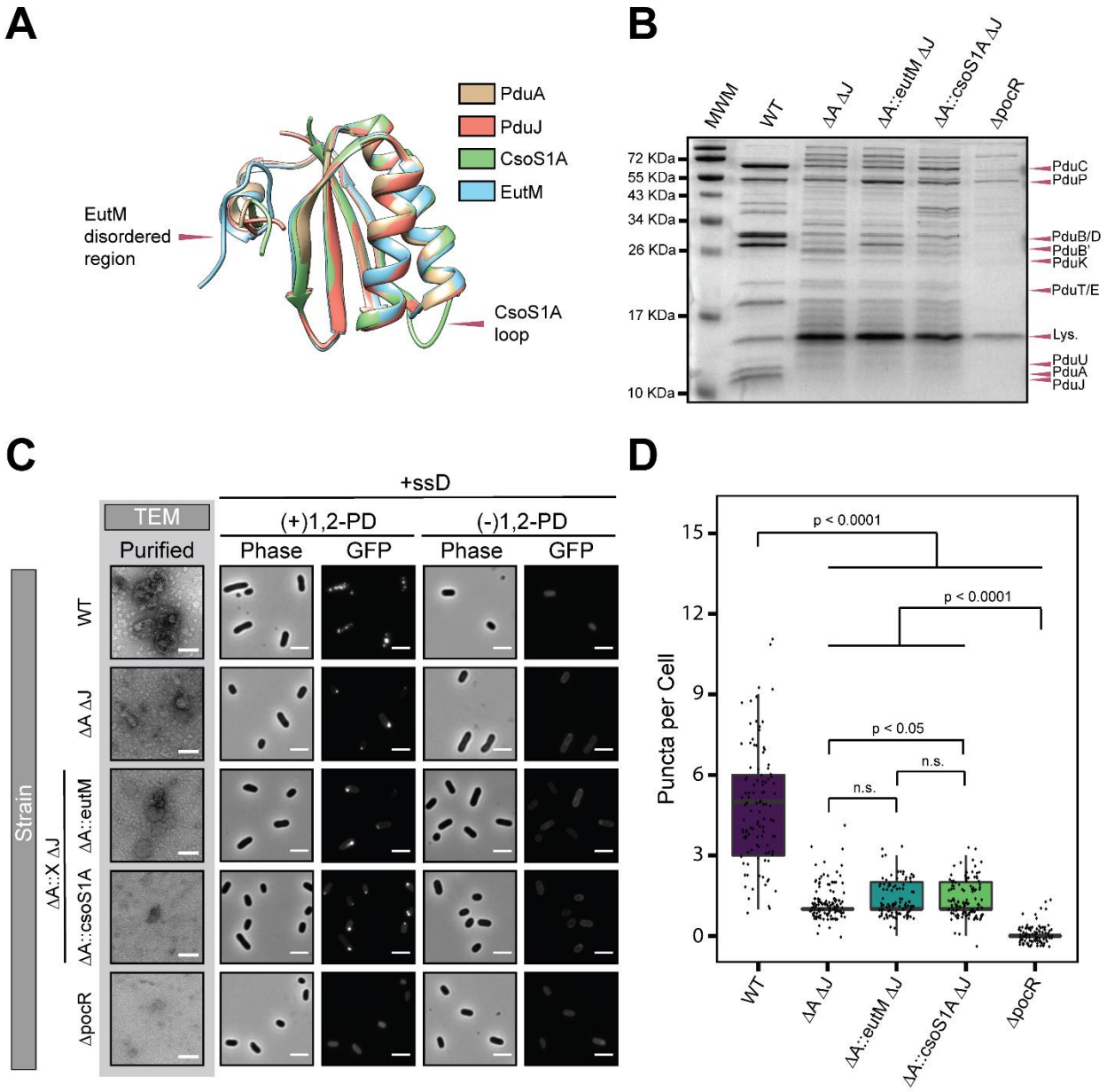
ΔA::J-K25A ΔJ
ΔpocR

**Figure 7**


A
PduA
PduJ
CsoS1A
EutM
EutM disordered region
CsoS1A loop
B
MWM
WT
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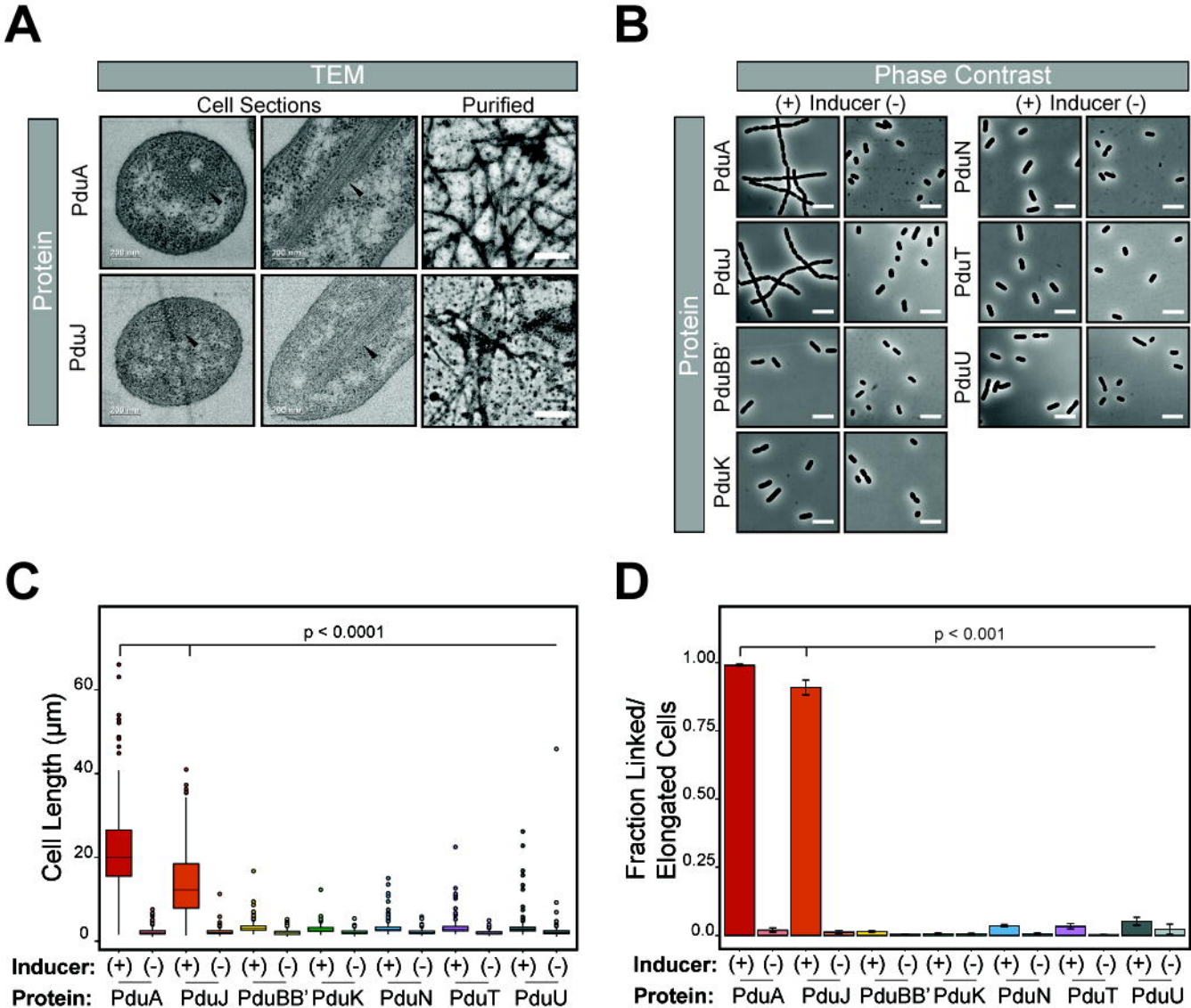
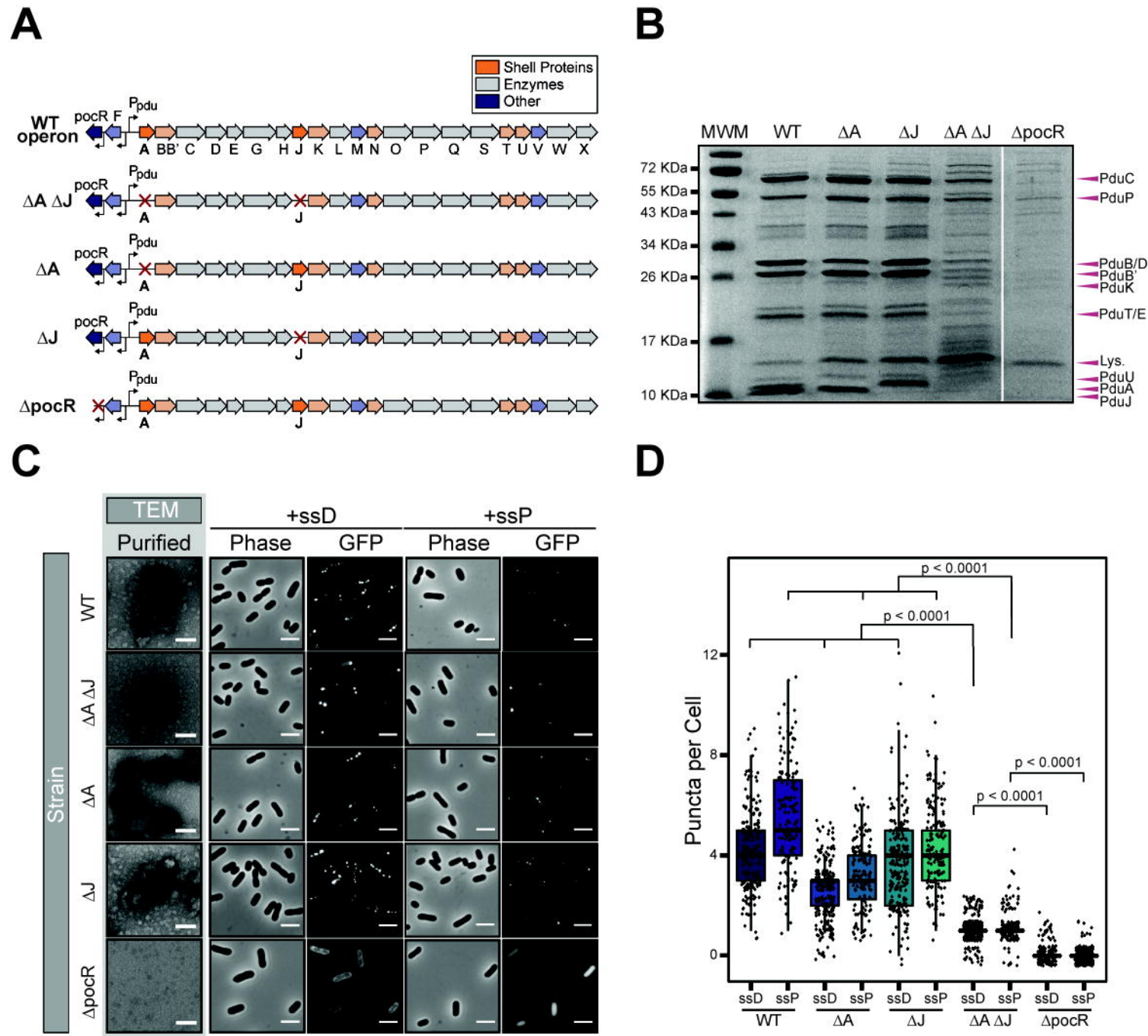
ΔA::csoS1A ΔJ
ΔpocR
72 KDa
55 KDa
43 KDa
34 KDa
26 KDa
17 KDa
10 KDa
PduC
PduP
PduB/D
PduB'
PduK
PduT/E
Lys.
PduU
PduA
PduJ
C
+ssD
TEM
Purified
(+)1,2-PD
(-)1,2-PD
Phase
GFP
Phase
GFP
Strain
ΔA::X ΔJ
WT
ΔA ΔJ
ΔA::eutM
ΔA::csoS1A
ΔpocR
D
Puncta per Cell
p < 0.0001
p < 0.0001
p < 0.05
n.s.
n.s.
0
3
6
9
12
15
WT
ΔA ΔJ
ΔA::eutM ΔJ
ΔA::csoS1A ΔJ
ΔpocR

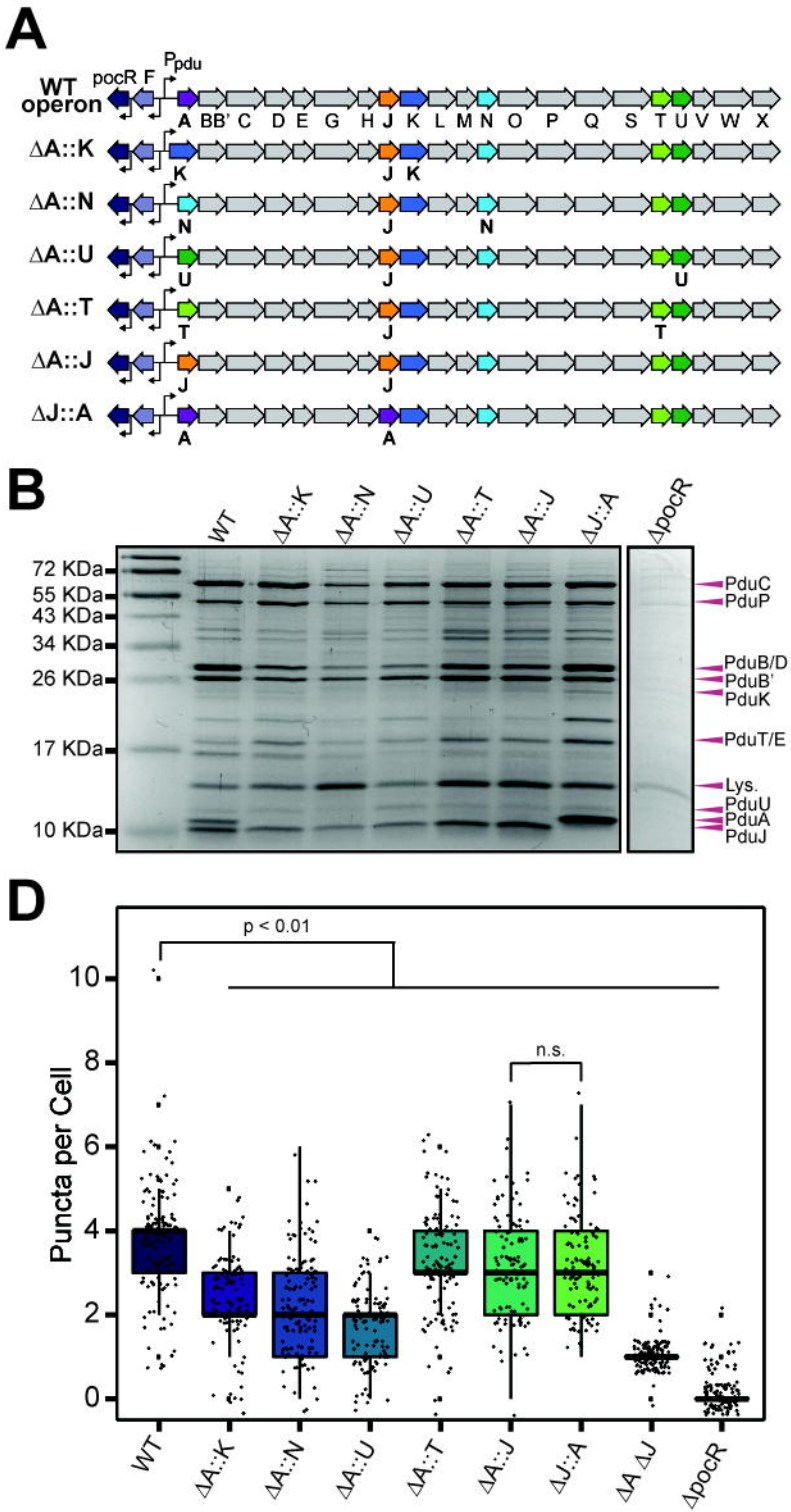
# Figure 1

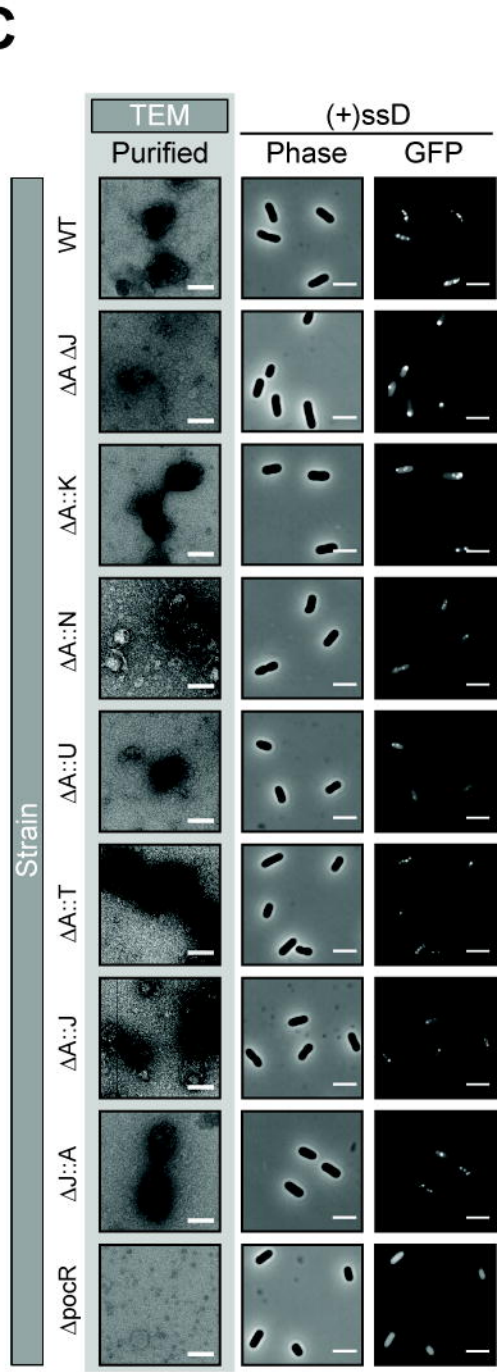

A
TEM
Cell Sections
Purified
Protein
PduA
PduJ
B
Phase Contrast
(+) Inducer (-)
(+) Inducer (-)
Protein
PduA
PduJ
PduBB'
PduK
PduN
PduT
PduU
C
p < 0.0001
Cell Length (µm)
60
40
20
0
Inducer: (+) (-) (+) (-) (+) (-) (+) (-) (+) (-) (+) (-) (+) (-)
Protein: PduA PduJ PduBB' PduK PduN PduT PduU
D
p < 0.001
Fraction Linked/ Elongated Cells
1.00
0.75
0.50
0.25
0.0
Inducer: (+) (-) (+) (-) (+) (-) (+) (-) (+) (-) (+) (-) (+) (-)
Protein: PduA PduJ PduBB' PduK PduN PduT PduU

# Figure 2

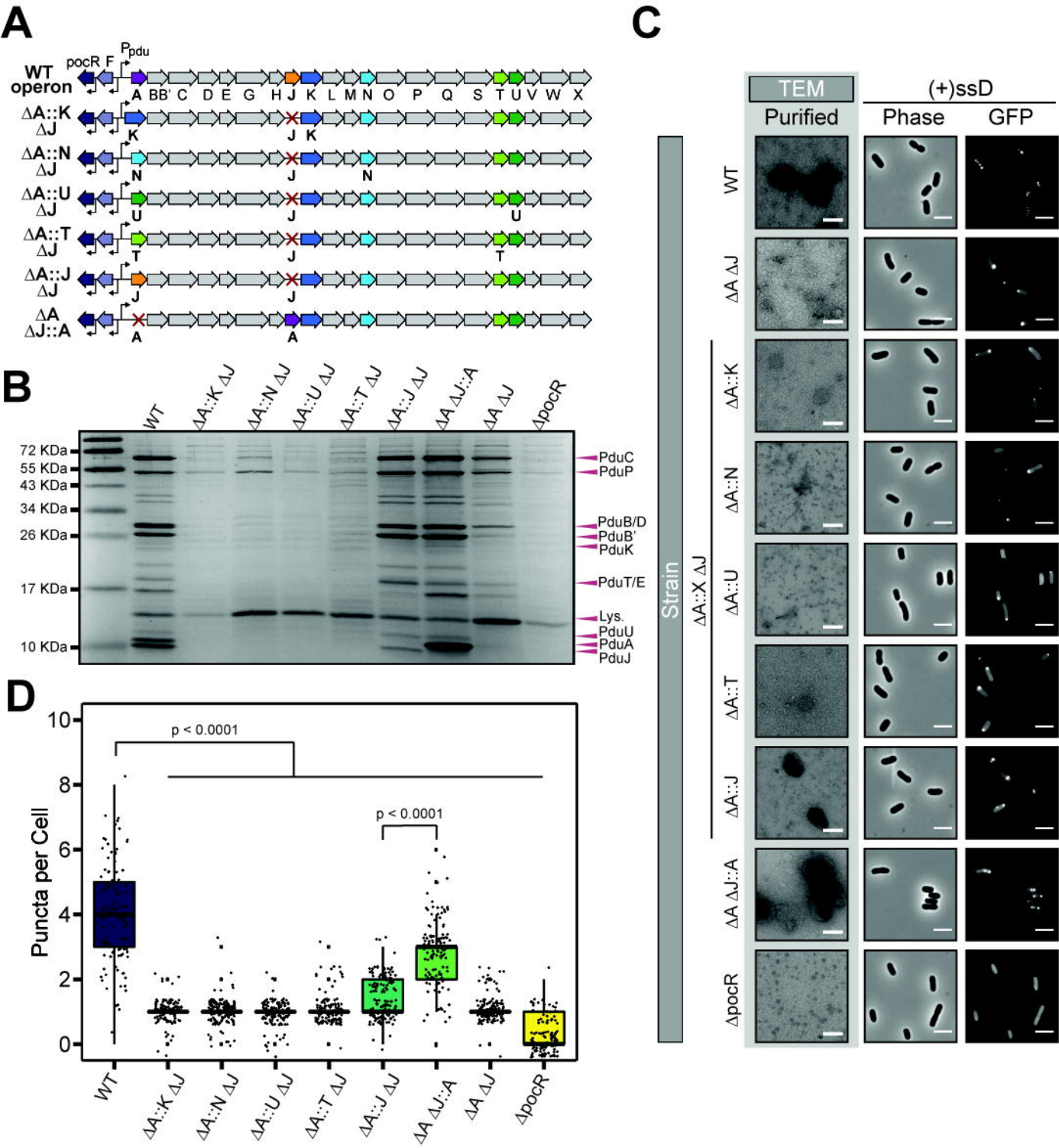

A
Shell Proteins
Enzymes
Other
WT operon
pocR F
Ppdu
A BB' C D E G H J K L M N O P Q S T U V W X
ΔA ΔJ
ΔA
ΔJ
ΔpocR
B
MWM WT ΔA ΔJ ΔA ΔJ ΔpocR
72 KDa
55 KDa
43 KDa
34 KDa
26 KDa
17 KDa
10 KDa
PduC
PduP
PduB/D
PduB'
PduK
PduT/E
Lys.
PduU
PduA
PduJ
C
TEM
Purified
+ssD
+ssP
Phase
GFP
Strain
WT
ΔA ΔJ
ΔA
ΔJ
ΔpocR
D
Puncta per Cell
p < 0.0001
ssD ssP
WT
ΔA
ΔJ
ΔA ΔJ
ΔpocR

# Figure 3

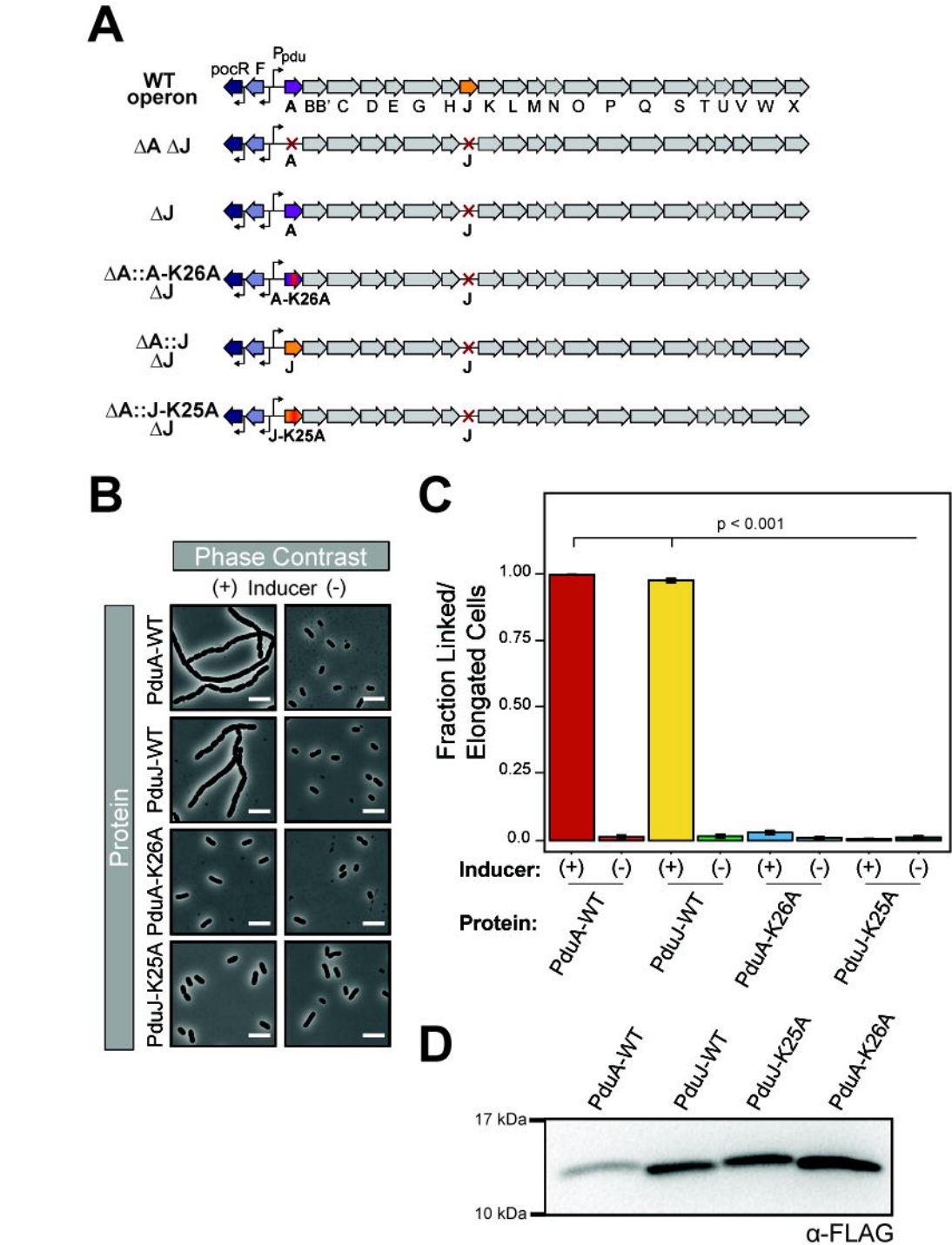

A
WT operon
pocR F
Ppdu
A BB' C D E G H J K L M N O P Q S T U V W X
ΔA::K
ΔA::N
ΔA::U
ΔA::T
ΔA::J
ΔJ::A
B
WT
ΔA::K
ΔA::N
ΔA::U
ΔA::T
ΔA::J
ΔJ::A
ΔpocR
72 KDa
55 KDa
43 KDa
34 KDa
26 KDa
17 KDa
10 KDa
PduC
PduP
PduB/D
PduB'
PduK
PduT/E
Lys.
PduU
PduA
PduJ
D
p < 0.01
n.s.
Puncta per Cell
0
2
4
6
8
10
WT
ΔA::K
ΔA::N
ΔA::U
ΔA::T
ΔA::J
ΔJ::A
ΔA ΔJ
ΔpocR


C
TEM
Purified
(+)ssD
Phase
GFP
Strain
WT
ΔA ΔJ
ΔA::K
ΔA::N
ΔA::U
ΔA::T
ΔA::J
ΔJ::A
ΔpocR

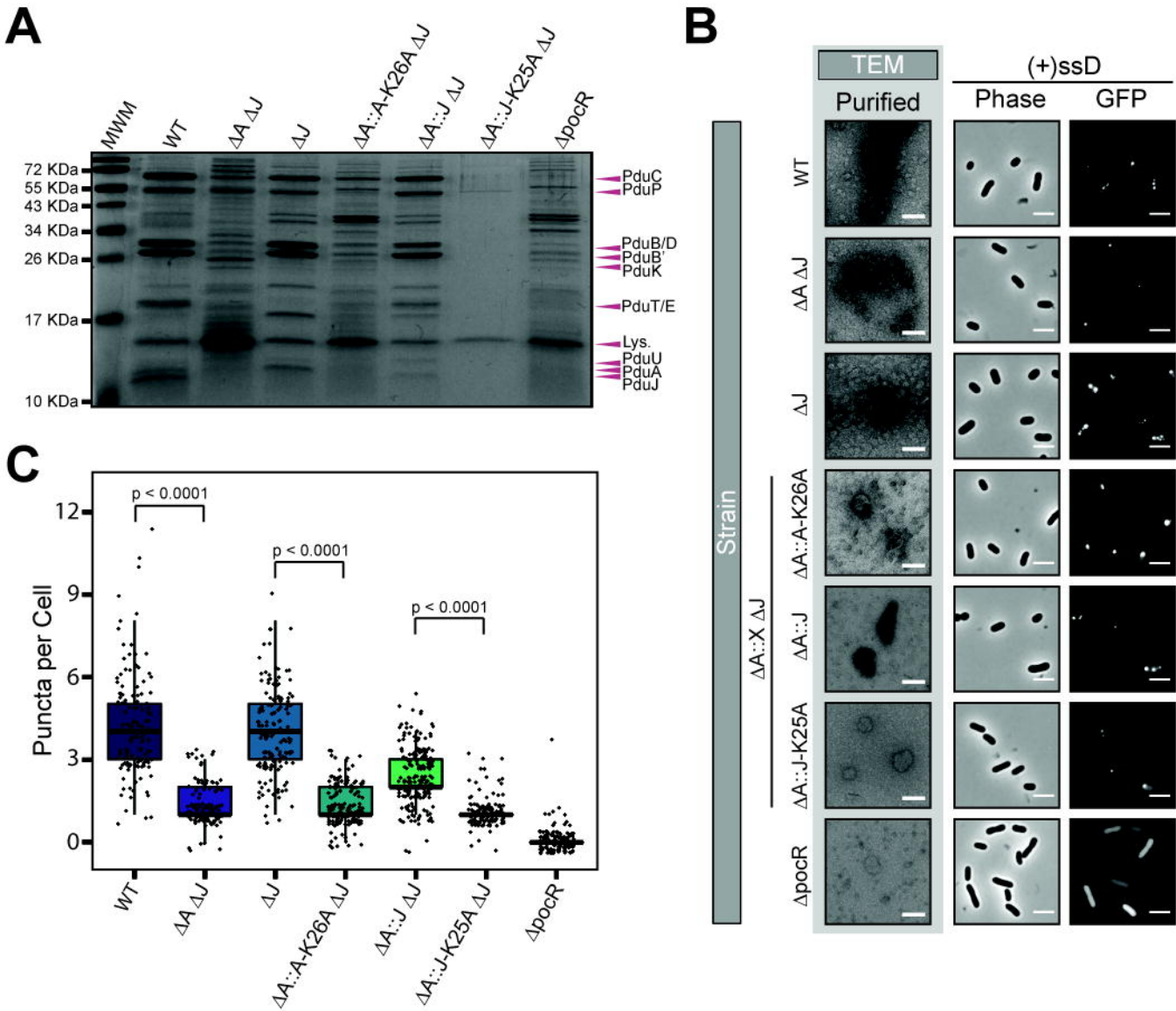
# Figure 4


A
pocR F
Ppdu
WT operon
A BB' C D E G H J K L M N O P Q S T U V W X
ΔA::K ΔJ
ΔA::N ΔJ
ΔA::U ΔJ
ΔA::T ΔJ
ΔA::J ΔJ
ΔA ΔJ::A
B
WT
ΔA::K ΔJ
ΔA::N ΔJ
ΔA::U ΔJ
ΔA::T ΔJ
ΔA::J ΔJ
ΔA ΔJ::A
ΔA ΔJ
ΔpocR
72 KDa
55 KDa
43 KDa
34 KDa
26 KDa
17 KDa
10 KDa
PduC
PduP
PduB/D
PduB'
PduK
PduT/E
Lys.
PduU
PduA
PduJ
C
TEM
Purified
(+)ssD
Phase
GFP
Strain
ΔA::X ΔJ
D
Puncta per Cell
p < 0.0001
p < 0.0001

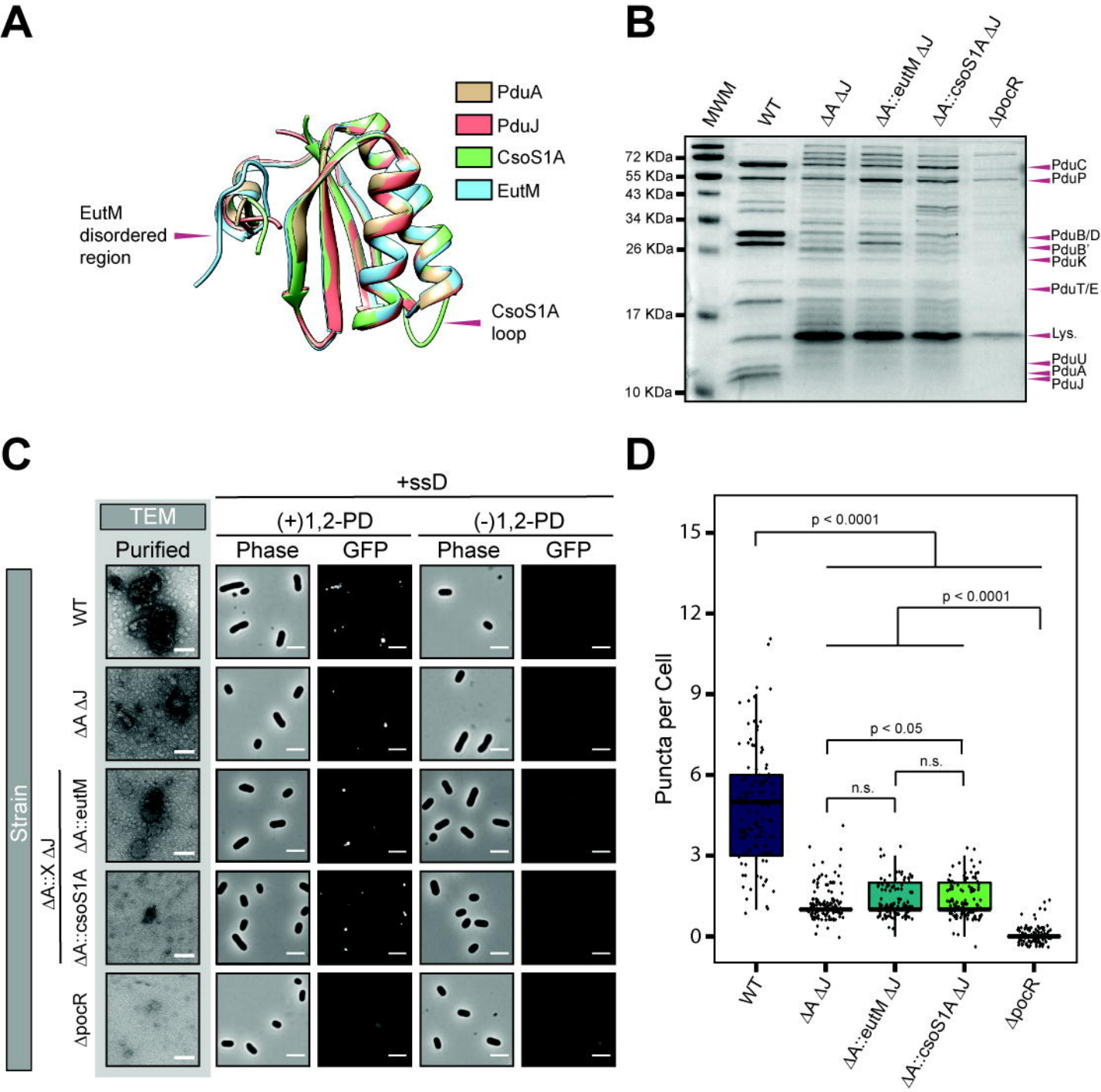
# Figure 5


A
WT operon
pocR F
Ppdu
A BB' C D E G H J K L M N O P Q S T U V W X
ΔA ΔJ
A
J
ΔJ
A
J
ΔA::A-K26A ΔJ
A-K26A
J
ΔA::J ΔJ
J
J
ΔA::J-K25A ΔJ
J-K25A
J
B
Phase Contrast
(+) Inducer (-)
Protein
PduA-WT
PduJ-WT
PduA-K26A
PduJ-K25A
C
p < 0.001
Fraction Linked/ Elongated Cells
1.00
0.75
0.50
0.25
0.0
Inducer: (+) (-) (+) (-) (+) (-) (+) (-)
Protein: PduA-WT PduJ-WT PduA-K26A PduJ-K25A
D
PduA-WT
PduJ-WT
PduJ-K25A
PduA-K26A
17 kDa
10 kDa
α-FLAG

# Figure 6


A
MWM
WT
ΔA ΔJ
ΔJ
ΔA::A-K26A ΔJ
ΔA::J ΔJ
ΔA::J-K25A ΔJ
ΔpocR
72 KDa
55 KDa
43 KDa
34 KDa
26 KDa
17 KDa
10 KDa
PduC
PduP
PduB/D
PduB'
PduK
PduT/E
Lys.
PduU
PduA
PduJ
B
TEM
Purified
(+)ssD
Phase
GFP
Strain
ΔA::X ΔJ
WT
ΔA ΔJ
ΔJ
ΔA::A-K26A
ΔA::J
ΔA::J-K25A
ΔpocR
C
p < 0.0001
p < 0.0001
p < 0.0001
Puncta per Cell
12
9
6
3
0
WT
ΔA ΔJ
ΔJ
ΔA::A-K26A ΔJ
ΔA::J ΔJ
ΔA::J-K25A ΔJ
ΔpocR

# Figure 7


A
PduA
PduJ
CsoS1A
EutM
EutM disordered region
CsoS1A loop
B
MWM
WT
ΔA ΔJ
ΔA::eutM ΔJ
ΔA::csoS1A ΔJ
ΔpocR
72 KDa
55 KDa
43 KDa
34 KDa
26 KDa
17 KDa
10 KDa
PduC
PduP
PduB/D
PduB'
PduK
PduT/E
Lys.
PduU
PduA
PduJ
C
+ssD
TEM
Purified
(+)1,2-PD
(-)1,2-PD
Phase
GFP
Phase
GFP
Strain
WT
ΔA ΔJ
ΔA::X ΔJ
ΔA::eutM
ΔA::csoS1A
ΔpocR
D
Puncta per Cell
p < 0.0001
p < 0.0001
p < 0.05
n.s.
n.s.
0
3
6
9
12
15
WT
ΔA ΔJ
ΔA::eutM ΔJ
ΔA::csoS1A ΔJ
ΔpocR