

Opposing Contributions of Polynucleotide Phosphorylase and the Membrane Protein NlpI to Biofilm Formation by *Salmonella enterica* Serovar Typhimurium[∇]

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CsgD and cyclic-3',5'-di-guanylate are key regulators of biofilm formation in *Salmonella enterica* serovar Typhimurium. Our results show that polynucleotide phosphorylase and NlpI oppositely altered expression of CsgD. Polynucleotide phosphorylase and NlpI also had opposite effects on the expression of *yjcC*, which codes for a cyclic-3',5'-di-guanylate phosphodiesterase affecting CsgD expression.

The ability to create multicellular communities in the form of a biofilm has gained increased appreciation as a prokaryotic response to selected environmental cues (3, 6, 8). In *Salmonella enterica* serovar Typhimurium and *Escherichia coli*, biofilm formation is initiated upon growth at a low temperature on solid media of low osmolarity (5, 7, 13). In the presence of the dye Congo red, this biofilm is characterized by a red, dry and rough (rdar) colony appearance (6). The rdar morphotype involves production of at least four extracellular matrix components, including CsgAB fimbriae (2, 6, 7).

Biofilm development in *S. Typhimurium* is a genetically highly regulated process that includes an array of key regulatory proteins (8) and the secondary messenger cyclic-di-guanylic acid monophosphate (c-di-GMP) (6, 8, 12). The intracellular level of c-di-GMP is spatially and temporally regulated by the activities of GGDEF and EAL domain proteins (8, 12). GGDEF and EAL domain proteins synthesize and degrade c-di-GMP, respectively (8, 12).

Polynucleotide phosphorylase (PNPase) (encoded by *pnp*) is an evolutionarily conserved enzyme affecting gene expression in bacteria, plants, and mammals (10). In *E. coli*, PNPase assists acclimatization to cold through its ability to degrade selected cold-induced transcripts (14). We have previously demonstrated that PNPase affects around 4% of the coding sequences in *S. Typhimurium* during growth in rich medium (15). Recently we noted that PNPase also contributes to growth at decreased temperatures and motility of *S. Typhimurium*. The expression of these phenotypes also required the membrane protein NlpI (S. F. Rouf et al., unpublished data). Here we demonstrate that PNPase and NlpI, in contrast, regulate rdar morphotype development in *S. Typhimurium* SR-11 oppositely.

rdar morphotypes of *pnp* and *nlpI* mutants. rdar morphotype development was assayed on Congo red Luria agar plates without salt at 28°C (12). Plates were inoculated with a 10- μ l drop of overnight cultures grown in Luria broth suspended in phosphate-buffered saline (PBS) to an optical density at 600 nm (OD₆₀₀) of 0.5. The rdar morphotype development was followed for up to 72 h. When necessary, plates were supplemented with antibiotics (ampicillin, 100 μ g/ml; chloramphenicol, 10 μ g/ml; and kanamycin sulfate, 50 μ g/ml; Sigma Aldrich). For induction of NlpI from the recombinant plasmid pSFR04 (cloned into pBAD33 under the *araC* promoter; S. F. Rouf et al., unpublished data), media were supplemented with 0.1% L-(+)-arabinose (Sigma).

Compared to that of the wild type, development of the rdar morphotype was delayed in the *pnp* mutant and enhanced in the *nlpI* mutant (Fig. 1A). The *pnp nlpI* double mutant appeared more rugous than the wild type but less rugous than the *nlpI* mutant (Fig. 1A). Complementation with the cloned *pnp* gene (pMC109) (1) restored the rdar morphotype in the *pnp* mutant. The cloned *nlpI* gene (pSFR04) suppressed biofilm development in the *nlpI* mutant (Fig. 1B).

Expression of CsgD and CsgA in *pnp* and *nlpI* mutants. CsgD is a master activator of biofilm formation through its ability to induce expression of the CsgA and CsgB fimbrial subunits and cellulose (3, 5, 9). We therefore compared the expression of CsgD and CsgA in the wild-type and mutant strains. CsgD was assayed by immunoblotting (12). Equal numbers of wild-type and mutant bacteria grown in LB without salt at 28°C for 20 h were solubilized in SDS-PAGE sample buffer. The proteins were separated on 12% SDS-PAGE gels (4) and transferred to polyvinylidene difluoride membranes (Hybond P; Amersham). Polyclonal rabbit anti-CsgD peptide antiserum (1:5,000) and horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (1:5,000; Pierce) were used as primary and secondary antibodies (7). Chemoluminescence signal intensities of CsgD were analyzed using the Quantity One software program (Bio-Rad). CsgA was extracted from bacterial cultures grown at 28°C for 20 h on LB agar without salt (12). CsgA was depolymerized in formic acid, and the CsgA monomers

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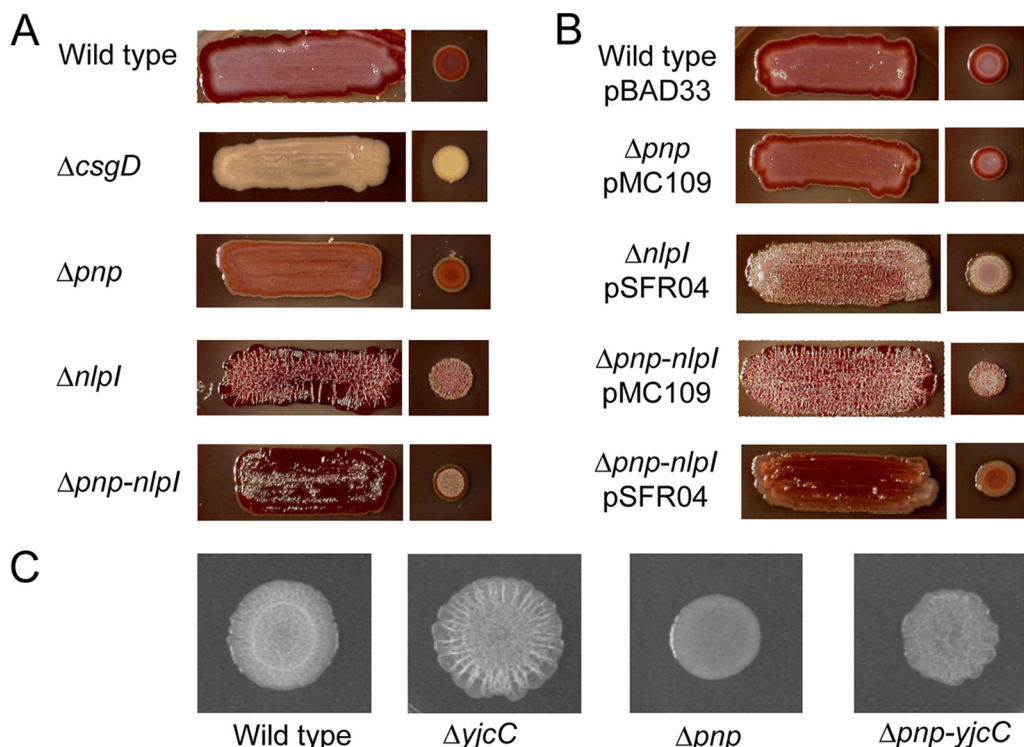


FIG. 1. Formation of the rdar morphotype in *S. Typhimurium* on Congo red plates. Plates were seeded as either streaks or drop-on lawns. Incubation was carried out at 28°C for 72 h, except for noncomplemented drop-on lawns, which were read after 48 h.

were analyzed on 12% SDS-PAGE gels (4) after staining with Coomassie blue.

In agreement with rdar morphotype development, expression of CsgD and CsgA was substantially reduced in the *pnp* mutant (Fig. 2, left panels). In contrast, the *nlpI* mutant showed increased expression of both CsgD and CsgA (Fig. 2, left panels). Quantification of immunoblot signals revealed a more than 10-fold difference in CsgD expression when the *pnp* and *nlpI* mutants were compared (Fig. 2, top left). The *pnp nlpI* mutant showed an intermediate level of CsgD expression (Fig. 2, top left).

To estimate *csgD* and *csgA* mRNA levels, total bacterial RNA was isolated using the TRI Reagent (Sigma) from cultures grown in LB without salt at 28°C. RNA samples were reverse transcribed for quantitative real-time PCR (qRT-PCR) (SYBR green Jumpstart; Sigma) (Table 1). The transcripts were quantified on an ABI Prism 7000 sequence detection system (Applied Biosystems). To assess qRT-PCR primer ef-

iciencies, the dissociation curve was analyzed for each primer pair. Each of these showed a single peak. Relative signals of the transcripts were analyzed using the *rfaH* gene as an endogenous control, since the expression of *rfaH* is unaffected by *pnp* (15). RNA samples that had not been reverse transcribed were used as negative controls for DNA contamination. In these analyses, the *csgD* and *csgA* mRNA levels closely followed the corresponding protein expression levels (Fig. 3).

Biofilm formation in *yjcC* mutants. The c-di-GMP synthetase *AdrA* and the phosphodiesterases *YhjH*, *YciR*, and *YjcC* all have an impact on rdar morphotype development (3, 5, 12). *YjcC* and *YciR* in particular suppress expression of CsgD (12). We therefore extended the qRT-PCR analysis to

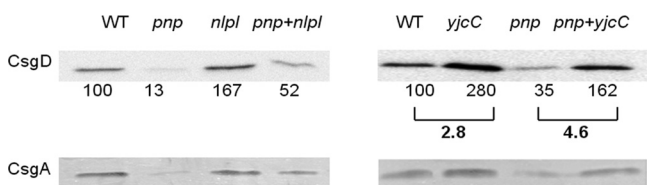


FIG. 2. Effects of *pnp* and *nlpI* mutations on CsgD and CsgA expression in *S. Typhimurium*. CsgD and CsgA expression was analyzed by immunoblotting with Coomassie blue-stained 12% SDS-PAGE gels. Relative signal intensities and the ratios thereof are shown for CsgD.

TABLE 1. Primers used for qRT-PCR

Primer	Sequence	Target gene
<i>FadrA</i>	TCGCTGGAAGTCACGCTCT	<i>adrA</i>
<i>RadrA</i>	CGCTTATGTTCCGCTAATTTAATG	<i>adrA</i>
<i>FcsgA</i>	CATCGACCAGTGGAAACGCTA	<i>csgA</i>
<i>RcsgA</i>	TTACGCTGGAATCAGATGCG	<i>csgA</i>
<i>FcsgD</i>	CCCTGACGATTATCCCTACCG	<i>csgD</i>
<i>RcsgD</i>	CCCTGTAATCCGCTGACCAC	<i>csgD</i>
<i>FrfaH</i>	GGCGTTGTGTCGATCCTGAAAC	<i>rfaH</i>
<i>RrfaH</i>	CGTGATGATGACGCTATCGC	<i>rfaH</i>
<i>FyciR</i>	GCGAATACAGACACCGTCACC	<i>yciR</i>
<i>RyciR</i>	ACGATACCCACCTGCGACTC	<i>yciR</i>
<i>FyjcC</i>	AAAAACGCGTCAGCATTTCAGT	<i>yjcC</i>
<i>RyjcC</i>	CCGCGCTCAGTCACTTCAA	<i>yjcC</i>
<i>FyhjH</i>	CTGCTGCTGCAACTGATGAAC	<i>yhjH</i>
<i>RyhjH</i>	CTCCACGCCCTCGACAAT	<i>yhjH</i>

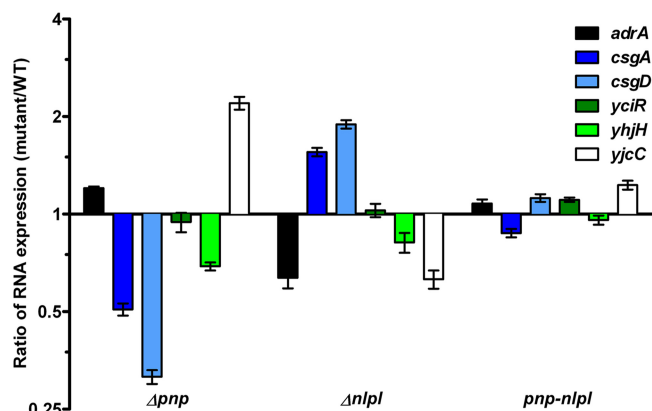


FIG. 3. Effect of *pnp* and *nlpI* mutations on gene expression in *S. Typhimurium*. Transcript expression ratios for *adrA*, *csgA*, *csgD*, *yciR*, *yhjH*, and *yjcC* mRNA levels as given for the *pnp*, *nlpI*, and *pnp nlpI* mutants in relation to those for the wild type at 8 h postinoculum.

include *adrA*, *yciR*, *yjcC*, and *yhjH*. The mRNA coding for the biofilm suppressor YjcC was markedly increased in the *pnp* mutant and was decreased in the *nlpI* mutant (Fig. 3). Furthermore, *pnp* and *nlpI* had minor, but inverse, effects on the expression of *adrA*. The expression levels of *yjcC*, *yhjH*, and *adrA* were restored to wild-type levels in the *pnp nlpI* mutant (Fig. 3).

To establish a connection between *pnp*, *yjcC*, and biofilm formation, we constructed a *yjcC* mutant and a *pnp yjcC* double mutant by P22 *int* phage transduction (11). Compared to that of the wild type, biofilm development was enhanced in the *yjcC* and *pnp yjcC* mutants (Fig. 1C). When CsgD expression was quantified by immunoblotting, the *yjcC* mutant revealed a 2.8-fold enhancement in CsgD expression (Fig. 2, top right). When the *yjcC* mutation was introduced into the *pnp* mutant, we noted a 4.6-fold enhancement in the expression of CsgD (Fig. 2).

Conclusion. Here we have demonstrated that PNPase and NlpI have opposite effects on the expression of CsgD, a key activator of biofilm formation. This observation is associated with a subsequent divergent effect of PNPase and NlpI on the expression of CsgA and the biofilm-associated rdar morphotype development. YjcC is a major phosphodiesterase suppressing CsgD expression (12). Expression of *yjcC* was increased in the *pnp* mutant and decreased in the *nlpI* mutant. The altered expression of *yjcC* became restored in the *pnp nlpI* double mutant. In parallel, we noted that the impact of YjcC on the expression of CsgD was more pronounced in the absence of PNPase. Therefore, the effect of PNPase and NlpI on biofilm formation could relate to their opposite effects on the expression of YjcC (Fig. 4). These results also bring forth an unrecognized gene regulatory connection between PNPase and c-di-GMP metabolism.

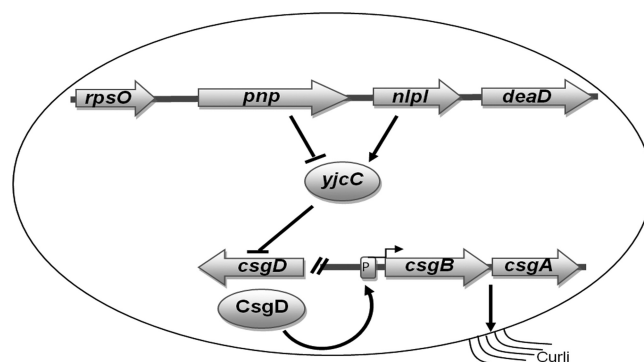


FIG. 4. Model for the concerted activity of *pnp*, *nlpI*, and *yjcC* in relation to the expression of *csgD*.

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