Highlight

Cyclic-di-GMP signaling meets extracellular polysaccharide synthesis in *Bacillus subtilis*

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Summary

In order to resist harmful environmental conditions, many bacteria form multicellular aggregates called biofilms. In these biofilms, they protect themselves in a self-produced matrix consisting of extracellular polysaccharides, proteins, and DNA. In many bacteria, biofilm formation is stimulated in the presence of the second messenger cyclic di-GMP. In this issue of *Environmental Microbiology Reports*, Bedrunka and Graumann have studied matrix production by the proteins encoded in the *Bacillus subtilis ydaJKLMN* operon. For the first time, they were able to provide a link between c-di-GMP signaling and matrix production in this bacterium. The work demonstrates that the c-di-GMP receptor protein YdaK forms a membrane-bound complex with the YdaM and YdaN proteins, and that this interaction with YdaK is required for polysaccharide production by YdaL, YdaM, and YdaN.

Bacteria can choose between a variety of different lifestyles to adapt to changing environmental conditions. Moreover, within populations, individual cells can decide for one or the other lifestyle. The Gram-positive model bacterium *Bacillus subtilis* can explore nutrients in liquid media as motile cell; alternatively, the bacteria can form multicellular structures called biofilms. In addition, *B. subtilis* may sporulate to resist enduring adverse conditions or become compentent to take up foreign DNA and thus to have the chance of acquiring novel valuable genetic information (Lopez and Kolter, 2010). The decision of individual cells or populations depends on environmental signals such as nutrient or ion availability as well as on intrinsic genetically encoded programs that allow apparently homogeneous cultures to differentiate (Lopez *et al*., 2009; Lopez and Kolter, 2010; Cairns *et al*., 2014).

In many bacteria, the motile and sessile lifestyles are mutually exclusive, *i. e*. a cell can either be motile and explore its environment, or it is part of a multicellular aggregation, the biofilm, in which cells are embedded in a common matrix and thus protected from harmful interactions with the environment. The decision for either lifestyle is made in each cell individually, and in many bacteria this decision is controlled by cyclic di-GMP, a second messenger. If the concentrations of c-di-GMP are high, the cells prefer biofilm formation whereas low concentrations of c-di-GMP are in favour of motility (Hengge, 2009; Jenal *et al*., 2017). In *B. subtilis*, there are three diguanylate cyclases that produce this second messenger (see Fig. 1) (Chen *et al*., 2012; Gao *et al*., 2013). However, physiological conditions that control the activities of these enzymes have not been established, and the intracellular concentration of the nucleotide is rather low under standard growth conditions (Gao *et al*., 2013; Diethmaier *et al*., 2014). As mentioned above, c-di-GMP is required for biofilm formation in many bacteria. Strikingly, biofilm formation is not affected by the deletion of all diguanylate cyclases in *B. subtilis* suggesting different mechanisms of signal transduction in this bacterium. In contrast, the major diguanylate cyclase CdgF is essential for biofilm formation in *Bacillus thurigiensis* (Fagerlund *et al*., 2016). Consistent with the c-di-GMP independent biofilm formation in *B. subtilis*, this enzyme does not have a counterpart in *B. subtilis*. Interestingly, another second messenger has been implicated in the control of biofilm formation in *B. subtilis*: accumulation of the essential second messenger cyclic di-AMP results in strongly reduced expression of the genes required for matrix production and, thus, to the inability of the bacteria to form biofilms (Gundlach *et al*., 2016). C-di-AMP is thought to be involved in cell wall homeostasis and the control of potassium uptake (Commichau *et al*., 2015), and the molecular mechanisms by which c-di-AMP interferes with biofilm formation are poorly understood.

The search for c-di-GMP targets has so far identified three proteins in *B. subtilis*: the PilZ domain protein DgrA, and the unknown proteins YdaK and YkuI (Chen *et al*., 2012; Gao *et al*., 2013). While DgrA and YkuI have been implicated in the control of motility and zinc homeostasis, respectively (Chen *et al*., 2012; Chandrangsu and Helmann, 2016), no function has been established for YdaK. In their study published in this issue of *Env. Microbiol. Reports*, Bedrunka and Graumann (2017) provide evidence that YdaK is involved in the control of extracellular polysaccharide biosynthesis.

Functional analysis of the *ydaJKLMN* operon

In biofilms, the bacteria are embedded in a self-produced matrix that consists of extracellular polysaccharides (EPS), proteins, and DNA (Cairns *et al*., 2014). In *B. subtilis*, the proteins encoded by the *epsA-O* operon synthesize the major EPS, poly-N-acetylglucosamine (Roux *et al*., 2015). In addition, the levansucrase encoded by *sacB* polymerizes the fructose moiety of sucrose to levan. As *sacB* is expressed only at very high sucrose concentrations (Crutz *et al*., 1990), levan synthesis is unlikely to contribute to biofilm matrix production under physiological conditions. Bedrunka and Graumann (2017) have identified a third gene cluster possibly involved in EPS synthesis. The *ydaJKLMN* operon is part of a genomic region that is present in standard laboratory strains of *B. subtilis* such as 168, but absent from other commonly used strains such as JH642. The bioinformatic analysis of each protein encoded in the operon suggests a function in EPS production and its control. The first gene of the operon, *ydaJ*, codes for a glycosyltransferase that is attached to the other side of the cell membrane (see Fig. 1). The evidence suggests that this protein by itself is not necessary for EPS production but that it may modify the polysaccharide. The second gene, *ydaK*, encodes a

membrane protein with a degenerate GGDEF domain at the C-terminus. While GGDEF domains are generally thought to be active in c-di-GMP synthesis, degenerate domains are not catalytically active but participate as c-di-GMP binding proteins in signal transduction (Jenal *et al*., 2017). Indeed, binding of c-di-GMP to YdaK has been demonstrated experimentally, although only at high concentrations of the second messenger (Gao *et al*., 2013). YdaM is similar to glycosyltransferases suggesting that the protein might be involved in EPS synthesis. Indeed, using a series of elegant genetic studies, the authors provide compelling evidence that EPS biosynthetic activity is encoded in the operon. The analysis of the contributions of the different genes revealed that YdaK, YdaL, YdaM, and YdaN are essential for EPS biosynthesis and complex colony formation resulting from it. As YdaK is a c-di-GMP binding signal transduction protein, it is likely that the YdaL, YdaM, and YdaN proteins provide the catalytic activities for EPS synthesis, and that YdaJ modifies the polysaccharide.

B. subtilis strains lacking the *epsA-O* operon or *epsH* mutants defective in the production of undecaprenyl-3-O-acyl N- acetylglucosamine are unable to produce extracellular polysaccharides and they are also defective in biofilm formation. This raises the question for the relevance of EPS production by YdaLMN. Indeed, the activity of the proteins encoded by the *ydaJKLMN* operon could only be detected upon artificial overexpression. However, the operon is controlled by the alternative sigma factor, σ^B, and only transcribed under stress conditions such as ethanol or oxidative stress. Thus, the matrix polysaccharide produced by YdaLMN is probably only synthesized if the bacteria experience such stress and may help the bacteria to protect themselves under harmful conditions.

c-di-GMP signaling and YdaLMN-dependent EPS synthesis

In their study, Bedrunka and Graumann have studied the localization of the proteins of the *ydaJKLMN* operon. YdaJ and YdaL are likely to be exposed to the outer side of the membrane and could therefore not be investigated by fluorescence microscopy. For YdaK, YdaM, and YdaN, they observed a similar pattern of intracellular localization: the proteins form static clusters at the membrane, especially at the cell poles. This membrane localization is in excellent agreement with a function in EPS biosynthesis. Strikingly, the three proteins co-

localize in these clusters suggesting that they form a complex and that the c-di-GMP receptor protein YdaK controls the activity of the enzymes in this complex (see Fig. 1). YdaK is required for the activity of the EPS biosynthetic machinery, and moreover, it needs to be inserted into the membrane in order to stimulate EPS biosynthesis by YdaLMN.

It is tempting to speculate that it is the c-di-GMP-bound form of YdaK, which stimulates

YdaLMN activity. This would place cyclic di-GMP in a central position in the lifestyle choice under stress conditions: c-di-GMP binding to the PilZ domain of the target protein DgrA results in an inhibitory interaction with the flagellar stator protein MotA and thus in inhibition of motility (Chen *et al*., 2012). On the other hand, binding of c-di-GMP to the degenerate GGDEF domain of YdaK stimulates YdaLMN activity and thus stress EPS biosynthesis. Thus, c-di-GMP might be crucial in achieving mutual exclusivity of motility and EPS production under stress conditions.

Today, we are just beginning to understand the molecular details of second messenger signalling in Gram-positive bacteria. The work presented by Bedrunka and Graumann paves new ways to approach this question.

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References

Bedrunka, P., and Graumann, P. L. (2017) Subcellular clustering of a putative c-di-GMP-dependent exopolysaccharide machinery affecting macro colony architecture in *Bacillus subtilis*. *Env Microbiol Rep* in press.

Cairns, L. S., Hobley, L., and Stanley-Wall, N. R. (2014) Biofilm formation by *Bacillus subtilis*: new insights into regulatory strategies and assembly mechanisms. *Mol Microbiol* **93**: 587-598.

Chandrangsu, P., and Helmann, J. (2016) Intracellular Zn(II) intoxication leads to dysregulation of the PerR regulon resulting in heme toxicity in *Bacillus subtilis*. *PLoS Genet* **12**: e1006515.

Chen, Y., Chai, Y., Guo, J. H., and Losick, R. (2012) Evidence for cyclic di-GMP-mediated signalling in *Bacillus subtilis*. *J Bacteriol* **194**: 5080-5090.

Commichau, F. M., Dickmanns, A., Gundlach, J., Ficner, R., and Stülke, J. (2015) A jack of all trades: the multiple roles of the unique essential second messenger cyclic di-AMP. *Mol Microbiol* **97**: 189-204.

Crutz, A. M., Steinmetz, M., Aymerich, S., Richter, R., and Le Coq, D. (1990) Induction of levansucrase in *Bacillus subtilis*: an antitermination mechanism negatively controlled by the phosphotransferase system. *J Bacteriol* **172**: 1043-1050.

Diethmaier, C., Newman, J. A., Kovacs, A. T., Kaever, V., Herzberg, C., Rodrigues, C., Boonstra, M., Kuipers, O. P., Lewis, R. J., and Stülke, J. (2014) The YmdB phosphodiesterase is a global regulator of late adaptive responses in *Bacillus subtilis*. *J Bacteriol* **196**: 265-275.

Fagerlund, A., Smith, V., Røhr, Å. K., Lindbäck, T., Parmer, M. P., Andersson, K. K., Reubsaet, L., and Økstad, O. A. (2016) Cyclic diguanylate regulation of *Bacillus cereus* group biofilm formation. *Mol Microbiol* **101**: 471-494.

Gao, X., Mukherjee, S., Matthews, P. M., Hammad, L. A., Kearns, D. B., and Dann, C. E. (2013) Functional characterization of core components of the *Bacillus subtilis* cyclic di-GMP signaling pathway. *J Bacteriol* **195**: 4782-4792.

Gundlach, J., Rath, H., Herzberg, C., Mäder, U., and Stülke, J. (2016) Second messenger signaling in *Bacillus subtilis*: accumulation of cyclic di-AMP inhibits biofilm formation. *Front Microbiol* **7**: 804.

Hengge, R. (2009) Principles of c-di-GMP signaling in bacteria. *Nat Rev Microbiol* **7**: 263-273.

Jenal, U., Reinders, A., and Lori, C. (2017) Cyclic di-GMP: second messenger extraordinaire. *Nat Rev Microbiol* **15**: in press.

Lopez, D., Fischbach, M. A., Chu, F., Losick, R., and Kolter, R. (2009) Structurally diverse natural products that cause potassium leakage trigger multicellularity in *Bacillus subtilis*. *Proc Natl Acad Sci USA* **106**: 280-285.

Lopez, D., and Kolter, R. (2010) Extracellular signals that define distinct and coexisting cell fates in *Bacillus subtilis*. *FEMS Microbiol Rev* **34**: 134-149.

Roux, D., Cywes-Bentley, C., Zhang, Y. F., Pons, S., Konkol, M., Kearns, D. B., Little, D. J., Howell, P. L., Skurnik, D., and Pier, G. B. (2015) Identification of poly-N-acetylglucosamine as a major polysaccharide component of the *Bacillus subtilis* biofilm matrix. *J Biol Chem* **290**: 19261-19272.

Figure Legend

Figure 1: Cyclic di-GMP signaling in *B. subtilis*.

Cyclic di-GMP can be synthesized from GTP by three distinct diguanylate cyclases and is degraded by the phosphodiesterase PdeH. The three c-di-GMP receptor proteins have distinct domains for the binding of the nucleotide. The formation of the complex between the c-di-GMP receptor YdaK and the EPS-synthesizing proteins YdaM and YdaN is essential for the catalytic activity of the latter proteins and for the synthesis of extracellular polysaccharides under stress conditions.