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REVIEW ESSAY

Prospects & Overviews

The many faces of the unusual biofilm activator RemA

Erhard Bremer[1] | Tamara Hoffmann[1] | Felix Dempwolff[1] | Patricia Bedrunka[1] | Gert Bange[1,2]

[1]Center for Synthetic Microbiology (SYNMIKRO), Philipps-University Marburg, Marburg, Germany

[2]Max Planck Institute for Terrestrial Microbiology, Marburg, Germany

Correspondence
Erhard Bremer and Gert Bange, Faculty of Chemistry and Center for Synthetic Microbiology (SYNMIKRO), Philipps-University Marburg, Karl-von-Frisch Strasse 14, 35043 Marburg, Germany.
Email: bremer@staff.uni-marburg.de; gert.bange@synmikro.uni-marburg.de

Abstract

Biofilms can be viewed as tissue-like structures in which microorganisms are organized in a spatial and functional sophisticated manner. Biofilm formation requires the orchestration of a highly integrated network of regulatory proteins to establish cell differentiation and production of a complex extracellular matrix. Here, we discuss the role of the essential *Bacillus subtilis* biofilm activator RemA. Despite intense research on biofilms, RemA is a largely underappreciated regulatory protein. RemA forms donut-shaped octamers with the potential to assemble into dimeric superstructures. The presumed DNA-binding mode suggests that RemA organizes its target DNA into nucleosome-like structures, which are the basis for its role as transcriptional activator. We discuss how RemA affects gene expression in the context of biofilm formation, and its regulatory interplay with established components of the biofilm regulatory network, such as SinR, SinI, SlrR, and SlrA. We emphasize the additional role of RemA played in nitrogen metabolism and osmotic-stress adjustment.

KEYWORDS

Bacillus subtilis, biofilms, DNA-binding, gene regulation, nitrogen metabolism, osmotic stress, transcriptional activator

INTRODUCTION

Most microorganisms live in biofilms, microbial assemblages encased in a self-produced extracellular matrix.[1–5] A biofilm is often regarded as a form of a multicellular organism,[6–8] where the microbial community possesses properties and characteristics not identifiable when one studies a given microorganism in isolation.[5] Biofilms can be found essentially in every niche on Earth occupied by microorganisms and can be formed on solid surfaces, at liquid-air/gas interfaces, and under conditions where the cells are completely submerged.[9] Biofilms pose considerable challenges for humans through their formation on medical devices (e.g., catheters) and through their increased resistance to anti-microbial agents.[10] On the other hand, biofilms can also be exploited in biotechnology for the production of commercially valuable compounds, in plant protection, wastewater treatment, and bioremediation.[11–13] Given the eco-physiological, medical and biotechnological importance of microbial biofilms, it is not surprising that considerable efforts have been made over the years to unravel the genetic and cellular events leading to and during biofilms formation, and those occurring during their dispersal.

Within the biofilm, genetically identical cells can form functionally distinct cell types.[14] A common denominator of microbial biofilms is their encasement in an extracellular polymeric substance matrix (EPS) that typically consists of polysaccharides, secreted proteins, and extracellular DNA.[1,15] This matrix forms the “house/city” of the cells that actively create the biofilm.[4,16,17] The EPS provides mechanical stability, contributes to water retention, and increases tolerance against disinfectants and antibiotics.[18] Cells encased in the EPS can take advantage of the close proximity to their neighbors through the acquisition, exchange, and recycling of nutrients and

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stress protectants.[19,20] The cells can benefit via division of labor during biofilm matrix production,[21] and by integrating their collective behavior through the synthesis of signaling molecules (e.g., quorum sensing)[22] and potassium-dependent electric currents,[23–25] processes that will also allow the timely production of virulence factors by pathogenic microorganisms.[21] Moreover, biofilms also facilitate horizontal gene transfer between the cells via conjugation, transduction and transformation, as biofilms cannot only be formed by members of a single microbial species but can also comprise multi-species assemblages.[26,27] Furthermore, different chemical (e.g., oxygen, water, salt, and nutrients) and mechanical gradients formed within the biofilm generate heterogeneous microenvironments leading to special cellular adaptation responses of the individual cell.[20,23] In contrast to single cells, a biofilm can be seen as a collection of highly differentiated microorganisms that cooperate for the common good of the population through interconnected cellular responses and developmental programs. However, there is also intense competition between members of the biofilm.[27,28]

BIOFILM-FORMATION BY *BACILLUS SUBTILIS*

Nondomesticated *Bacillus subtilis* isolates as model systems for biofilm formation

One of the microorganisms that has been intensively studied in biofilm research is *B. subtilis*, a Gram-positive, spore-forming bacterium ubiquitously found in terrestrial and marine ecosystems.[29,30] Ecophysiologically, the ability of *B. subtilis* to form biofilms on plant roots[31,32] provides the cells with access to a rich supply of nutrients present in root exudates and osmostress relieving compounds.[33,34] Biofilm formation on roots is also a major trait required for the ability of *B. subtilis* to promote plant growth and protect plants from disease-causing microorganisms.[31,32,35]

In 1877, Ferdinand Cohn reported the isolation and initial microbiological characterization of *B. subtilis*.[36] Already in this description, Cohn pointed to the multicellular nature of *B. subtilis* cultures, an attribute which he carefully documented in the drawing of this hallmark publication.[7,36] However, the knowledge about the multicellular nature of *B. subtilis* was not widely appreciated, as the approach used by bacterial geneticists to study the physiology and development of this spore-forming microorganism required the isolation of "well-defined" single colonies. As a consequence, *B. subtilis* got domesticated to laboratory conditions, where the formation of "sticky" colonies (= producing the biofilm relevant EPS) was a rather undesirable trait for genetic analysis.[14] Because of this evolution in the laboratory, all *B. subtilis* strains widely used for physiological and genetic studies[37] accumulated mutations that negatively affected, or even abrogated, biofilm formation.[38–40] Hence, it was widely assumed that *B. subtilis* cannot form biofilms.

Game changing was the approach taken by the groups of Roberto Kolter and Richard Losick (both at Harvard University, USA) when they introduced a nondomesticated isolate of *B. subtilis*, the ancestral strain NCIB3610, as a model system for biofilm formation.[38] This isolate, and related strains more readily accessible to genetic analysis,[41] can form structured macrocolonies on solid surfaces, and film-like structures at liquid-air interfaces (Figures 1A, B). The idea that nondomesticated *B. subtilis* isolates[39,41,42] were well-suited for biofilm research, gave the field an enormous boost, scientific progress that has been lucidly described in several truly outstanding reviews.[14,21,43–46] This allows us to only briefly describe here the core elements of the genetically highly complex regulatory circuits shaping biofilm formation in *B. subtilis*. Into this context of cellular differentiation by *B. subtilis* into biofilms, we can now place the crucial role of the transcriptional activator protein RemA,[47,48] whose crystal structure has recently been elucidated.[49]

B. subtilis biofilm formation: Core regulatory circuits and cellular physiology

Three loci (*epsA-epsO*, *tapA-sipW-tasA*, *bslA*) are at the core of biofilm formation of *B. subtilis* as they encode the enzymes and proteins to produce the extracellular matrix.[14,21,43,44,46] The 15 genes comprising the *epsA-epsO* operon encodes the enzymes for the synthesis of the exopolysaccharide component of the matrix.[38] The *tapA-sipW-tasA* operon encodes an extracellular protein (TasA) that forms long fibers, while TapA serves as an anchoring and assembly protein for TasA. SipW is a type I signal peptidase, processing the N-terminal signal peptides of both TapA and TasA during their secretion across the cytoplasmic membrane.[50] BslA, the product of the monocistronic *bslA* gene, is a biofilm surface layer protein that functions as a hydrophobin.[14,43,45]

Crucial to the cellular differentiation of *B. subtilis* is the response regulator protein Spo0A,[51] a transcription factor present all the time. Spo0A can be phosphorylated (Spo0A∼P) by various kinases to promote DNA-binding. Five histidine kinases with partially overlapping functions mediate the phosphorylation of Spo0A. Four of these (KinA, KinB, KinC, KinD) are embedded in the cytoplasmic membrane (Figure 1C), while the remaining kinase (KinE) is a cytoplasmic protein. The output generated by these kinases is transmitted through a multi-protein comprising phosphorelay to Spo0A.[51,52] The degree of Spo0A phosphorylation varies in response to environmental and cellular cues,[53] which allows Spo0A∼P to determine cell fate[14,44] Low levels of Spo0A∼P induce the expression of operons encoding toxins allowing *B. subtilis* to turn into a cannibal to secure unusual food sources,[54] while intermediate levels trigger biofilm formation.[53] High levels of Spo0A∼P promote formation of desiccation- and stress resistant spores. Sporulation is the measure of last resort to ensure the long-term survival of the *B. subtilis* population when the nutrient supply has been completely exhausted.[14,43,55,56]

No single Kin kinase is solely responsible for biofilm formation as their individual contribution to this differentiation process changes in response to varying growth conditions.[57–59] The cues triggering biofilm formation are not completely understood, but KinC plays an import role in this process.[57,60,61] Its kinase activity is regulated by surfactin, a motility-related lipopeptide produced by *B. subtilis* and also other bacteria.[62] Surfactin inserts into the cytoplasmic membrane

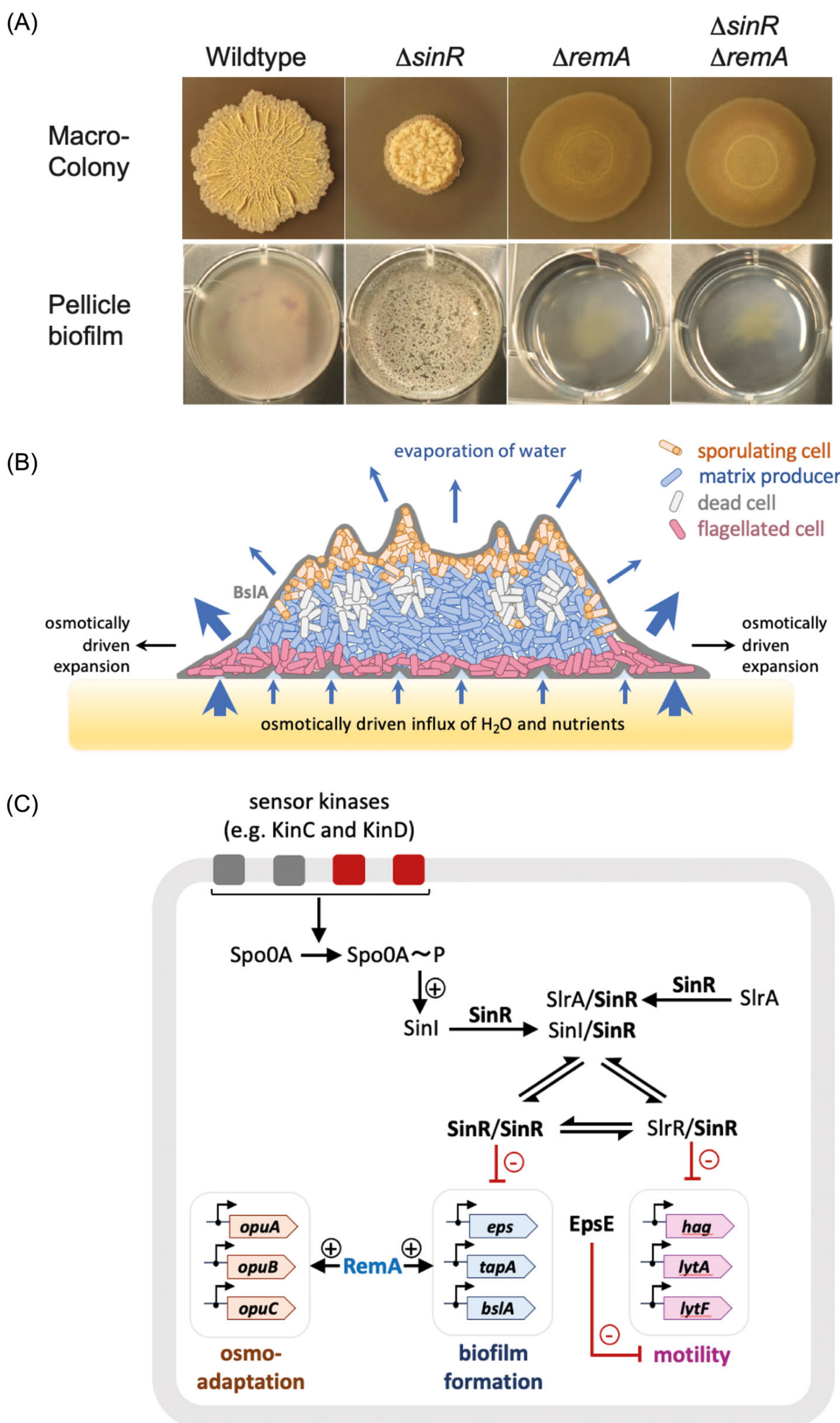

FIGURE 1 Architecture of the *B. subtilis* biofilm and the influence of the RemA transcriptional regulator on its formation. (A) Biofilm formation on solid surfaces and liquid-air interfaces. 10-μl of liquid pre-cultures of the undomesticated *B. subtilis* strain NCIB3610 and its mutant derivatives were spotted either onto the surface of MSgg agar plates[38] or used to inoculate MSgg liquid medium distributed in 6-well micro-plates. The biofilm-forming cells were grown for three days at 30°C and were then imaged with a digital reflex camera (D5600, Nikon). The following isogenic set of *B. subtilis* strains were used to visualize the formation of macrocolonies and pellicles: NCIB3610 [*remA*$^+$ *sinR*$^+$],[38] DS2679 ([*remA*::TnYLB *neo*^{R}] *sinR*$^+$),[47] DS859 [*remA*$^+$ *sinR*::*kan*^{R}],[71] TMB468 (*remA*::TnYLB *neo*^{R}) *sinR*::*spc*^{R}) (T. Hoffmann). (B) Scheme of a trans-sectional cut through a biofilm macro-colony and osmotically driven water fluxes influencing biofilm expansion. This figure was adapted with permission from a figure originally published by Bremer and Krämer (2019).[108] (C) Simplified overview of the regulatory network underlying biofilm formation in *B. subtilis*. The core genetic elements of *B. subtilis* biofilm formation and the influence of the RemA transcriptional activator protein on cellular

and thereby causes leakage of the major cellular cation, potassium. This process stimulates KinC activity and thus results in increased cellular Spo0A~P levels.[58,59] In turn, through its DNA-binding activity, Spo0A~P enhances the production of SinI, an antagonist of the SinR repressor. SinR is the central regulator of biofilm formation in *B. subtilis*.[63] SinR represses the transcription of the operons/genes (*epsA-epsO*, *tapA-sipW-tasA*, and *bslA*) required for the production of the extracellular matrix through its binding to the respective operators (Figure 1C). Increased SinI production leads to the sequestration of the SinR repressor into a SinI/SinR complex unable to bind DNA. As a consequence, biofilm formation is promoted (Figure 1C).[14,43,44]

However, SinI is not the only antagonist of SinR. SlrR is also able to interact with SinR with high affinity, and as such is thought to act similar to SinI. Moreover, the SlrR/SinR complex is able to bind DNA and to repress the transcription of genes required for motility and cell division (Figure 1C).[64] Therefore, the cellular level of SlrR is critical for the decision of whether a cell is motile or sessile, a prerequisite for biofilm formation.[64–67] Besides SinI and SlrR, SlrA seems also to act as a repressor of SinR, albeit the functional consequences are less clear.[64,65] Taken together, biofilm formation by *B. subtilis* relies on a highly tunable signaling and response network, which enables the integration of multiple external and internal cues with SinR in its center.[14,43] However, as shown in previous work,[63] and as visualized in Figure 1A, deletion of the *sinR* repressor gene does not abrogate biofilm formation by *B. subtilis*. This leaves open the possibility that other factor(s) are needed as positive activator(s) for biofilm formation once SinR is removed.

RemA: A CRUCIAL PLAYER IN BIOFILM FORMATION

Discovery of RemA

Onset of biofilm formation by *B. subtilis* is hallmarked by a cellular switch from a motile state to a sessile cell form that can adhere to various types of surfaces.[14,32,35,43] Motile cells are characterized by flagella, which are rotary motor structures enabling the movement of *B. subtilis* through liquids and on semisolid surfaces.[68,69] This process is influenced by the EpsE protein that not only functions as a glycosyltransferase participating in EPS synthesis,[43] but also restricts rotation of the flagellar motor.[70–72] Because SinR represses *epsA-epsO* transcription,[63,73,74] increased EpsE synthesis in a *sinR* mutant indirectly leads to nonmotile *B. subtilis* cells.[70,71]

Daniel B. Kearns and his team (Indiana University, USA) set up a suppressor screen to search for second-site mutants that can overcome the EpsE-mediated block in cell motility. Using transposon mutagenesis, two genetically unlinked insertion mutations in previously uncharacterized *B. subtilis* genes were recovered that were named *remA* and *remB* (*rem*: ***r****egulators of* ***e****xtracellular* ***m****atrix*).[47] RemA is a phylogenomically broadly conserved 89-amino-acid protein, while RemB is a more narrowly conserved 81-amino-acid protein. Disruption of either gene reduced the transcription of the *epsA-epsO* operon in a *sinR* mutant strain and thereby decreased the cellular levels of the motility inhibitor EpsE to a degree that the flagellum-dependent cell motility was restored. Concomitantly, biofilm formation was abrogated (Figure 1A).[47,48] In addition to the *epsA-epsO* and *tapA-sipW-tasA* operons, expression of *bslA*, the structural gene for the BslA hydrophobin,[75,76] is also under the control of the RemA.[48] Importantly, *remA* mutations are epistatic to a genetic disruption of *sinR*. In other words, even when the central biofilm operons *epsA-epsO* and *tapA-sipW-tasA* are expressed at high levels in the absence of the SinR repressor, RemA is strictly required for biofilm formation both on solid surfaces and at liquid-air interfaces (Figure 1A).[47] Hence, there can be no doubt that RemA serves as a central transcriptional activator protein for the expression of those genes that are at the core of biofilm formation by *B. subtilis*.

Microarray expression studies suggested further regulatory roles of RemA for the cellular physiology of *B. subtilis*.[48] By comparing the transcriptional profile of cells with an intact *remA* gene with that of an isogenic *remA* mutant strain, 70 genes were found to be affected by the loss of RemA. Of these, transcription of 51 genes were activated by RemA, while that of 19 genes was inhibited (see Tables 1 and 2 in reference[48]). Among those genes whose transcriptional activity was inhibited by RemA were twelve genes that are functionally associated with motility and chemotaxis, including *hag*, the structural gene of flagellin.[69,72] Notably, all these twelve genes are members of the σ^D-regulon.[77] Three functionally related groups could readily be identified among those 51 genes that required RemA for optimal genes expression. Besides the 14 genes associated with biofilm formation, the products of 11 genes are involved in the development of osmotic stress resistance, and 26 genes are associated with nitrogen metabolism (see Table 1 in reference [48] and our text below).

Although RemA was discovered more than 10 years ago, its genetic, biochemical, and structural analysis lags far behind in comparison with other key components of the regulatory network controlling biofilm formation by *B. subtilis*.[14,43,44] This is rather surprising because RemA is the only regulator of biofilm formation in *B. subtilis* that is essential for activating this cellular differentiation process. The recent report on the structural and biochemical analysis of RemA[49] now provides new incentives to further explore the role of this central biofilm regulator in *B. subtilis*. Moreover, genome-wide transcriptional data suggest a wider role for RemA in cellular physiology of *B. subtilis*,[48] thereby opening new avenues for research.

differentiation and osmotic tolerance. The genetic and biochemical regulatory circuits leading to the switch from planktonic motile cells to biofilms have been detailed in previously reported comprehensive reviews.[14,43,44] Cellular adaptation of *B. subtilis* to high osmolarity surroundings can occur through the import of various types of osmostress protectants (e.g., glycine betaine, carnitine) via the OpuA, OpuB, and OpuC ABC transport systems,[110] and RemA positively affects the transcription of the corresponding operons.[48]

RemA: a transcriptional activator with unique structural features

RemA is a small protein of only 89 amino acids with no apparent DNA binding motif, or domain, that can be deduced from its amino acid sequence.[47,48] Key to the successful structure determination of RemA by X-ray crystallography was the use of the homologue from the thermophilic bacterium *Geobacillus thermodenitrificans*, as biochemical and structural analysis of the recombinant *B. subtilis* protein was hampered by its low solubility.[49] In solution, RemA forms an octamer in which each of the eight subunits assembles in a donut-like structure (Figure 2A). Depending on protein concentration, both in solution and within the crystal, two RemA octamers can assemble into a dimeric superstructure, giving the visual impression of a double-donut (Figure 2B). A positively charged outer surface is created by three closely spaced arginines on each monomer of the RemA double-donut structure. (Figures 2A, B). Removal of the side chains of these arginines through site-directed mutagenesis to alanine residues abolished DNA-binding and the functionality of RemA as an activator for biofilm formation.[49]

The structural analysis of RemA showed that two adjacent subunits within the octamer possess significant structural similarity in its presumed DNA-binding region to LytTR-type DNA-binding domains (Figure 2C). These motifs are frequently found in multidomain, two component response regulators.[78–80] LytTR domains typically bind to specific DNA sites through the major groove side of their target DNA.[78] Superimposition of the structure of a DNA-bound LytTR domain present in the C-terminus of the response regulator AgrA (AgrA_C) from *Staphylococcus aureus* with a dimer of RemA gives a first impression of how RemA could bind DNA. This structural comparison shows that the surface-exposed arginines in the loop region of RemA[49] match well with the position of DNA bound by the LytTR-domain present in AgrA[78] (Figure 2C). The exact mechanism of DNA binding to RemA awaits further clarification. However, one RemA octamer might accommodate up to four DNA-binding motifs at the same time, which would than lead to a wrapping of the DNA around the RemA octamer (Figure 2D).

The DNA-binding capacity of RemA could further increase because, as mentioned above, two RemA octamers can form a functionally relevant double donut superstructure (Figures 2B, D).[49] In this configuration, RemA would be able to recognize up to eight DNA-binding motifs. Consequently, this would lead to a DNA-wrapping around the RemA octamer in a pattern similar to that observed for DNA wrapping in archaeal/eukaryotic nucleosomes (Figure 2E),[81–83] or in a fashion resembling that suggested for microbial AsnC/LrpC-type regulatory proteins (Figure 2F).[84–86].

The idea that RemA wraps DNA is supported by the observation that it binds to unusually long DNA targets ranging from 246 up to 290 base pairs. These DNA segments contain consecutive, but weakly conserved consensus sequences that are AT-rich.[48] The length of the DNA targets would be sufficient to wrap twice around the double donut superstructure of RemA (Figures 2B, D). The AT-rich nature of the DNA targets of RemA is important to enable a maximal degree of DNA bending, because AT-rich DNAs are well known for their bending abilities.[87] Thus, it is becoming evident that the RemA protein forms octameric rings that can assemble into a superstructure formed by two such rings.[49] RemA requires a series of individual, direct repeat binding sites with a weak consensus (AGNAAAA), which should cooperatively enable the binding of RemA to its target DNA.[48]

The structural analysis of RemA predicts eight DNA binding sites when two RemA octamers form a double donut structure (Figure 2B).[49] However, this prediction is not met by DNAse foot printing analysis at the P*eps*, P*tap*, and P*opu* promoters, safely revealing only six protected regions (Figures 3A, C).[48] The reason for this discrepancy might simply be the result of the N-terminal maltose binding protein (MBP)-tag used to increase solubility of the RemA protein used for the in vitro foot printing analysis.[48] We could recently show that tags present at N-terminus of the protein affect its ability to form its double donut superstructure,[49] which consequently would affect the number of observable footprints. Hence, future structural studies are needed to further clarify the exact details of the unusual way of DNA binding by RemA-type transcription factors. It is most urgent in this context to obtain a crystal structure of RemA with bound target DNA.

Integration of RemA into the transcriptional regulatory circuits shaping biofilm formation

How is the DNA-binding activity of RemA regulated? Loss of the SinR repressor derepresses the expression of the genes central for biofilm formation.[63,73,88] Despite this, RemA is strictly required for this cellular differentiation process, as a *remA* mutation is epistatic to a *sinR* mutation (Figure 1A).[48] This finding establishes the critical role of RemA for the entire biofilm forming process, and points to an interplay between SinR and RemA. In vitro transcription assays employing σ^A-containing RNA polymerase (RNAP) holoenzyme and purified RemA as a hybrid maltose-binding protein fusion consolidated this idea.[48] These experiments show that SinR can abolish the RemA-dependent transcription of P*eps* and P*tapA* promoters when present on linear templates. Therefore, SinR can be viewed as an anti-activator of RemA.[48]

RemA and SinR share overlapping binding motifs at the P*epsA* and the P*tapA* regions (Figure 3A)[48] In both cases, the weakly conserved RemA binding sites, which were identified by DNaseI protection assays,[48] partly overlap with the highly defined DNA recognition motifs of SinR (Figure 3A). At both promoters, two separately spaced SinR operators are present. To accommodate this finding, it has been suggested that the dimeric SinR protein might form a tetramer that could lead to loop formation of the target DNA (Figure 3B).[74,88] When SinR binds at the promoter of the *epsA-epsO* operon, the −10 and −35 promoter core elements could possibly still allow interactions with RNAP, while they are probably prevented through a DNA loop potentially formed by SinR at P*tapA* (Figure 3A). The known RemA and SinR binding sites differ between the P*epsA* and the P*tapA* promoter regions (Figure 3A; *compare the upper and lower panels*). While the positions of

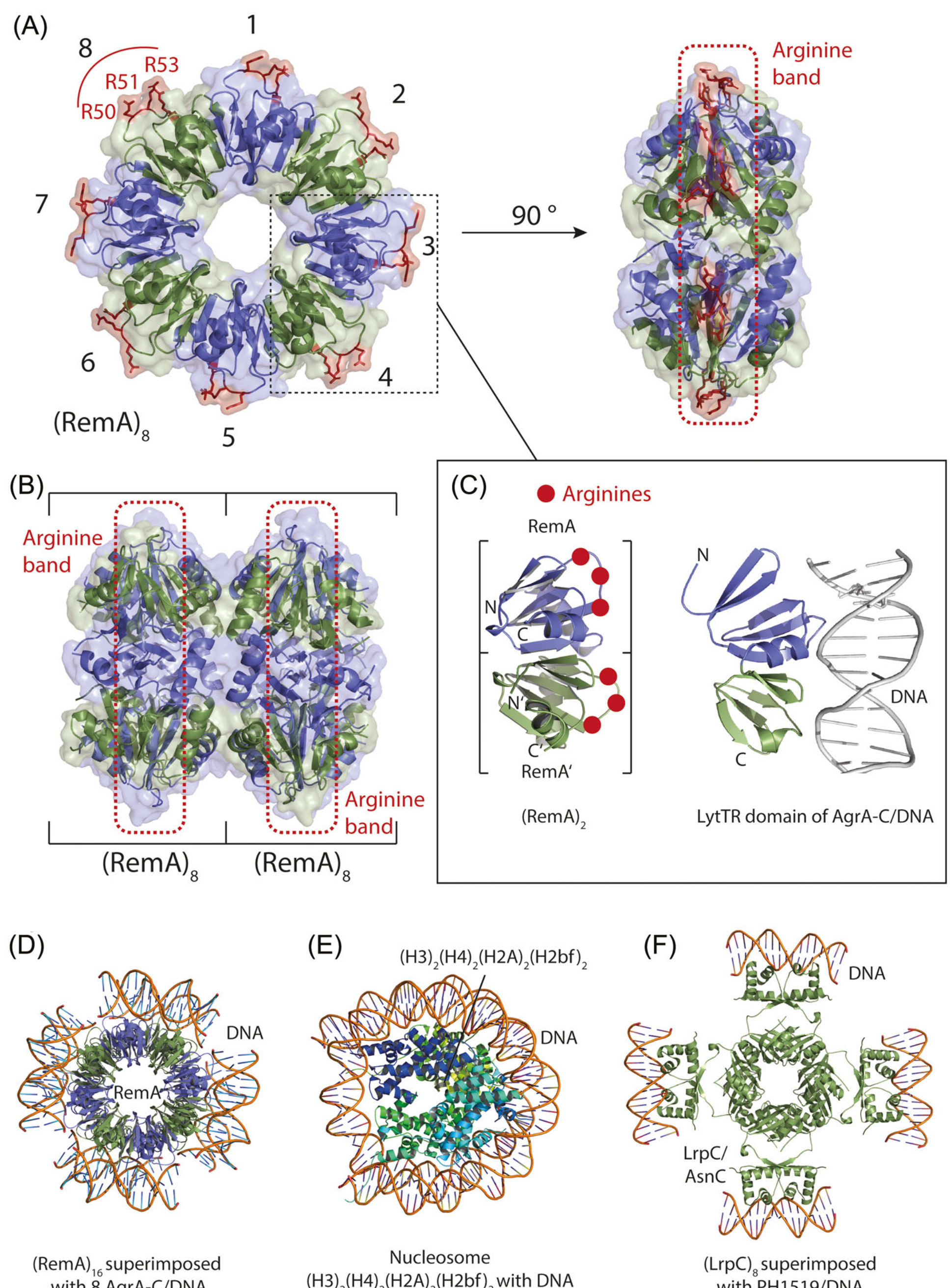

FIGURE 2 RemA is an unusual DNA binding protein. (A) Two views on the structure of the RemA octamer, which forms a donut-like structure.[49] The eight monomers within the octamer are alternately colored in blue and green. The positions of the arginines 50, 51, and 53, which are required for DNA binding,[49] are marked in red. (B) Structure of the dimer of the RemA octamer. The color code is as described in Figure 2A. (C) The structural arrangement of two monomers (*left side*) is reminiscent to the LytTR-type DNA-binding domain found in a variety of bacterial transcriptional regulators,[78,79] such as those found at the C-terminus of the AgrA response regulator from *S. aureus*[78] (*right side*). The positions of the three arginines required for DNA binding by RemA[49] are indicated by red dots. (D) Superimposition of the structures of eight AgrA C-terminal domains bound to DNA (PDB-ID: 3BS1) onto $(RemA)_{16}$ (PDB-ID: 7BM2). The AgrA-C domains were excluded from the figure for reasons of clarity; only the DNA (orange) is shown. (E) Structure of the nucleosome with the histone proteins (different colors) bound to DNA (orange) (PDB-ID: 2F8N). (F) Structure of the AsnC-type transcriptional regulator LrpC from *B. subtilis* (green; PDB-ID: 2CFX) superimposed with the PH1519 domain bound to DNA (PDB-ID: 2E1C). The PH1519 domain was excluded from the figure for reasons of clarity; the DNA is shown in orange

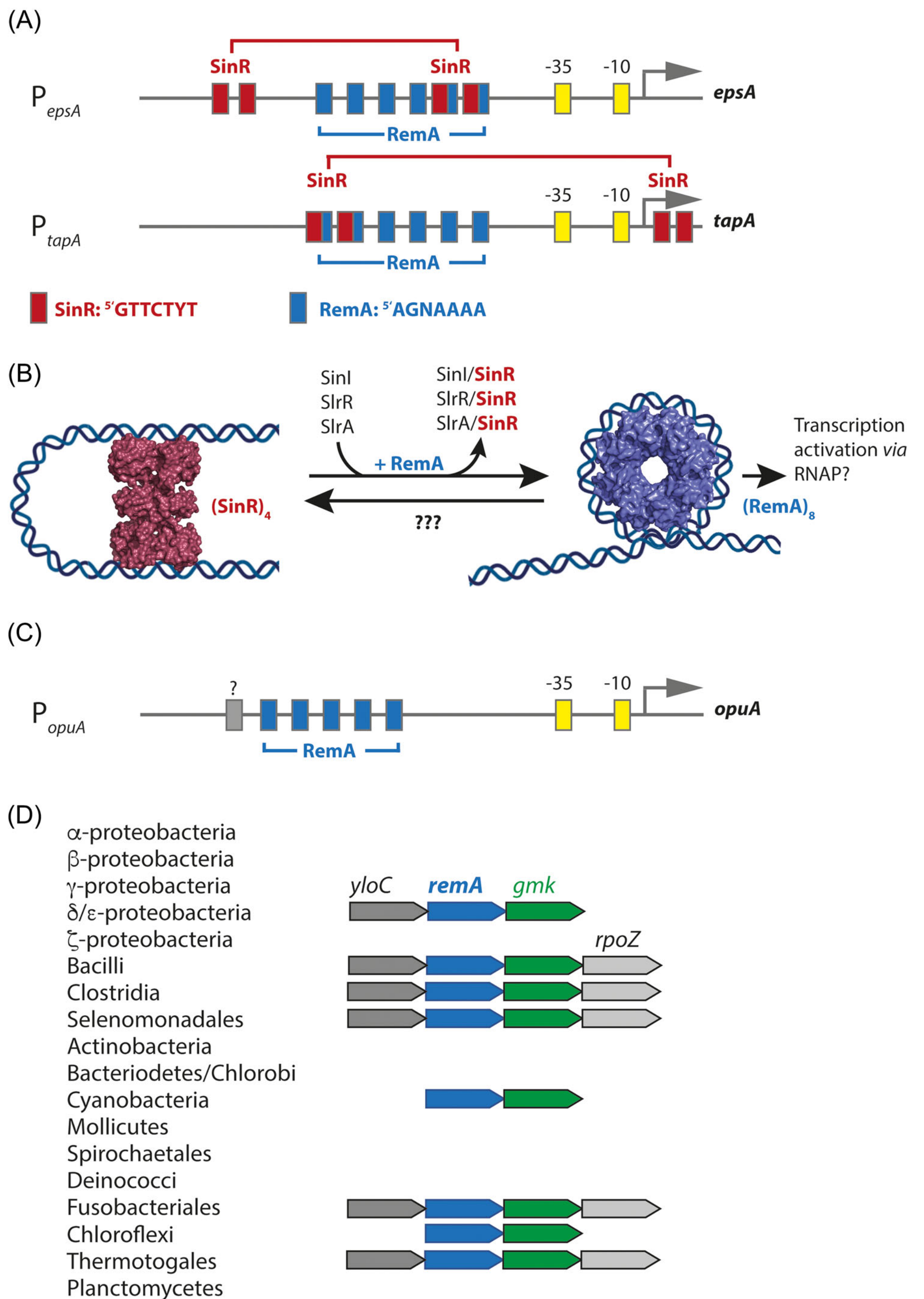

FIGURE 3 Transcriptional activation by RemA. (A) Scheme of the P*epsA* and P*tapA* promoter regions. The positions of RemA, SinR, and the −35 and −10 promoter regions are indicated in blue, red, and yellow, respectively. "Y" represents an unspecified pyrimidine base. (B) Hypothetical mechanism of the regulatory interplay of SinR (red) and RemA (blue). Note: The DNA is drawn as a cartoon and is only thought to illustrate different topologies, which we speculate to be induced by RemA and SinR. (C) Scheme of the P*opuA* regulatory region indicating RemA binding motifs in blue and the −35 and −10 boxes of the promoter are shown in yellow. The grey box indicates the binding site of a yet to be identified hypothetical regulator of RemA binding, which might act like the SinR repressor. (D) Organismic distribution and genetic neighborhood of *remA* (blue). *yloC* (dark grey) encodes for an endonuclease,[120] *gmk* (green) encodes for guanylate kinase converting GMP into GDP in an ATP-consuming manner,[118] and *rpoZ* (light grey) encodes for the omega subunit of the RNA polymerase.[119]

binding sites for RemA and SinR localize upstream of the −35 box of the σ^A-type P*epsA* promoter, the positions of two of the SinR binding sites are found downstream of the −10 box at P*tapA*. The functional consequences of the differences in the location of the binding sites are unknow. However, inspection of the positions of SinR and RemA binding sites at the P*epsA* and the P*tapA* promoter regions suggests that molecular differences exist in the interplay of RemA and its anti-activator SinR. The only experimental evidence supporting this idea is that SinR abolished all RemA-dependent protections and enhancements during the DNaseI footprint analysis at P*epsA*, while that was not the case for P*tapA*.[48] These experiments might suggest that SinR is able to occlude RemA from the P*epsA* promoter, while eventually being able to bind simultaneously with RemA at P*tapA*.

How might RemA and its anti-activator SinR act together at the molecular level? SinR interacts as a tetramer with its target DNA through its N-terminal Helix-turn-Helix (HTH) domain (Figure 3B, *left side*).[74,88,89] The HTH-domain of SinR inserts into the major groove, and SinR can make base-specific interactions with a maximum of five bases out the seven base-pairs long consensus motif.[74] In contrast, RemA forms octamers and a double octameric superstructure; it requires a series of individual, direct repeat binding sites with a weak consensus (AGNAAAA). This feature of its specific target sites is perhaps compensated for by cooperative binding of RemA.[48,49] Moreover, structural and biochemical evidence suggests that RemA is able to bend and wrap DNA in a way similar to nucleosomes (see above; Figure 3B, *right side*). These two highly different ways of DNA-binding used by the RemA and SinR proteins might lead to an altering of the DNA topology of the given promoter region, and as such change the expression of the respective genes.[90,91] However, further studies are required to critically assess this idea.

SinR, which seems to be constitutively produced at low levels throughout growth, is regulated by its antagonist SinI. SinI is expressed at low levels during vegetative growth and at higher levels during sporulation, because it is under the control of the sporulation response regulator, Spo0A.[92,93] However, upon activation of Spo0A, a subset of cells within the population expresses *sinI* at much higher levels,[64] thereby leading to biofilm formation. Besides SinI, SlrR also acts as repressor of SinR,[94,95] and the SlrR/SinR complex represses genes involved in flagellar biosynthesis and cell separation.[64,65] Moreover, SlrA also seems to act as a antagonist of SinR. Taken together, these data suggest that the DNA-binding of the SinR repressor is tightly regulated via these three antagonists (Figures 1C and 3B). This creates a complex molecular framework between RemA, SinR and its antagonists, as well as genetic feedback loops fostered by these interactions.[64–67,95] However, without RemA, this complex regulatory network leading to biofilm formation is going to fail, because RemA seems to ensure the “correct” DNA topology to activate the respective promoters for the operons/genes at the core of biofilm formation by *B. subtilis* (Figure 1C). How RemA executes this important function is subject to future research. Moreover, it is important to understand how the RemA-induced hypothesized topological changes of its DNA targets impacts transcription activation through RNAP. Furthermore, we need to understand whether other regulatory factors are involved, and whether the action of RemA might differ at different target promoters. Finally, it is unclear whether, when and how RemA releases its target DNA and whether this process is driven by concentrations of “free” SinR, or additional factors (Figure 3B).

How is DNA-binding of RemA or target gene selection regulated?

In *B. subtilis*, *remA* is expressed from two promoters. One of these is positioned up-stream of the *yloC-remA-gmk-rpoZ* gene cluster, while the second promoter is present in front of *remA*.[47,96] Notably, the transcription of *remA* is not under SinR control, ruling out the possibility that the epistatic nature of *remA* mutations over those of *sinR* gene disruptions is caused by transcriptional effects.[47] Data derived from a comprehensive genome-wide transcriptional profiling of *B. subtilis* cells grown under a wide spectrum of conditions suggest that the expression of the *remA* structural gene does not strongly vary.[96,97] These findings suggest that the RemA protein is probably present in rather constant levels in planctonic and sessile cells. Hence, the transcriptional control of *remA* does not seem to be the major factor shaping its role in biofilm formation.[47,48] Collectively, the available transcriptional data pointedly raise the question how the timing of DNA-binding by RemA, and hence transcriptional activation of biofilm genes, is determined, and how the selection of its target genes is accomplished. Currently, one can only speculate about the underlying molecular determinants for these processes, but posttranslational modifications, binding of low molecular mass effector molecules, and interactions of RemA with other proteins come to mind.

FUNCTIONS OF RemA BEYOND THE BIOFILM

Roles of RemA in nitrogen metabolism

A surprisingly large number of members (26 genes) of the RemA regulon are functionally associated with nitrogen metabolism as revealed by transcriptional profiling of *remA*$^+$ and *remA* mutant strains.[48] Prominent RemA-responsive operons were those for the import and utilization of purine bases (e.g., *pucA-E* and *pucJ-L*) that can be used by *B. subtilis* either for nucleotide synthesis or as source of nitrogen. The *pucA-E* and *pucJ-LM* operons are jointly controlled by the PucR regulatory protein.[98] Likewise, nitrate and ammonium transporter genes, such as *nasA*, and those for the nitrate reductase are members of the RemA-regulon as well.[48,99] Furthermore, the transcriptional activity of the gene encoding the L-asparaginase AnsZ, which converts L-asparagine into aspartate and ammonia,[100] was activated by RemA as well.[48] This was also true for genes encoding urease,[48] an enzyme which enables *B. subtilis* to use urea as alternative nitrogen source.[101] Notably, the transcriptional data derived from the RemA microarrays[48] correlate nicely with the comprehensive transcriptional profiling study of Nicolas *et al*. (2012), where all the aforementioned genes are highly expressed in *B. subtilis* cells grown in the

biofilm-promoting MSgg medium.[97] Taken together, these data imply that RemA plays an important role in activating genes central to the nitrogen metabolism of *B. subtilis*. However, to this end no further molecular or genetic data are available. It should be noted in this context that gene expression underlying the nitrogen stress response of developing *B. subtilis* strains are contributors to organize cellular differentiation with the biofilm in space and time.[102]

Adjusting to high osmolarity incurred cellular stress

Bacterial biofilms present a special osmotic environment for each participating cell, because the sugar components of the EPS generates osmotic pressure and affect water distribution, retention and the ability of the biofilm to repair structural disruptions.[45,103–107] The senor-kinase KinD (Figure 1C) is involved in detecting matrix components, and as a result of its signaling activity negatively affects matrix gene expression at high osmolarity.[103] Furthermore, the air-exposed surface of the *B. subtilis* biofilm is covered by the hydrophobin BslA, thereby endowing it with Teflon-like attributes (Figure 1B).[45,75,76] Consequently, these physical cues modulate and shape the growth of the entire biofilm,[104,105] and require osmostress adaptative responses by individual cells.[108,109] An important observation from the transcriptional profiling experiments[48] was therefore that RemA can also act at osmotically regulated promoters of operons encoding central components (OpuA, OpuB, OpuC) of the cellular *B. subtilis* osmostress adjustment response.[109,110] The *opuA*, *opuB*, and *opuC* operons encode ABC-transporters[110] for the import of a broad range of osmostress protectants (e.g., glycine betaine), the so-called compatible solutes.[108,111] These organic osmolytes are widely found in the soil and are present also in plant root exudates.[33,34,112] Their accumulation stimulates growth of *B. subtilis* under osmotically unfavorable environmental conditions.[109,110] Osmotic stress can originate from ionic (e.g., NaCl, KCl), but also from non-ionic compounds, such as sugars, which are highly abundant in the biofilm matrix.[113] For yet unknown reasons, sugars are particular strong inducers of *opu* gene expression when compared with iso-osmotic solutions of ions.[114]

RemA has so far only been studied at the osmostress-responsive promoter of *opuA*, encoding the major importer for the compatible solute glycine betaine in *B. subtilis*.[110] RemA binds in a repeating pattern at the osmoregulated σ^A-type *opuA* promoter,[114] as observed for the P*epsA* and the P*tapA* promoter regions (Figure 3C).[48] However, in comparison with the corresponding regulatory regions of P*epsA* and the P*tapA*, the mapped RemA binding sites at P*opuA* are positioned further upstream of the −35 region (compare: Figures 3A and C).[114] Binding of SinR to the P*opuA* has so-far not been reported, raising the question whether a yet unidentified repressor for the genetic control of the *opuA* promoter exists (Figure 3C). While no system-specific repressor or activator for *opuA* is known, GbsR-type repressors, members of the MarR-family, control the expression of the *opuB* and *opuC* operons.[115–117] Collectively, these findings suggest that RemA plays an important – yet poorly understood role – during the osmostress response of *B. subtilis* through its influence on osmotically regulated operons (*opuA*, *opuB*, *opuC*) encoding high-affinity importers for a wide spectrum of growth-promoting compatible solutes.[109,110] While RemA has been shown to bind to the P*opuA* regulatory region,[48] its influence on the genes for the major osmostress protectant import systems of *B. subtilis*[109,110] requires further molecular and physiological studies specifically in the framework of biofilm formation.[110] Furthermore, the question whether RemA serves as a general osmoregulatory transcription factor coordinating the cellular response by *B. subtilis* to high osmolarity surroundings needs to be answered as well.[108,109]

CONCLUSIONS AND FUTURE DIRECTIONS

RemA plays an essential role for biofilm formation of *B. subtilis*, but the importance of this transcriptional activator has long been overlooked. This unusual DNA-binding protein can form octamers with the ability to form a dimeric superstructure. As such, RemA has the potential to wrap DNA around its positively charged outer surface and thereby create nucleosome-like structures. These structures will alter local DNA topology and hence should affect gene expression. While a basic understanding for the role of RemA in controlling EPS production is emerging, the molecular underpinnings for its role in osmostress adaptation and nitrogen metabolism are still elusive.

In all bacteria possessing *remA*, this gene always colocalizes with *gmk* encoding guanylate kinase (Figure 3D), a central enzyme of the guanosine-triphosphate (GTP) biosynthesis pathway.[118] However, the physiological relevance of the conserved *remA-gmk* genetic architecture is unclear but it should be noted that these two genes are co-expressed in *B. subtilis*[48] along with *rpoZ*, the omega-subunit of the RNA-polymerase.[119] Moreover, *remA* is present in many bacteria,[48] albeit no studies on its function exist outside of *B. subtilis*. Interestingly, some of the species that possess *remA*-type genes do not possess identifiable matrix genes in their sequenced genomes,[48] implying a wider, yet to be fully defined, role of the RemA regulatory protein in microbial physiology.

We expect that the recently elucidated structure of the RemA transcriptional activator will serve as a starting point for further mechanistic studies of this protein and its multifaceted roles in *B. subtilis* and beyond.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

AUTHORS CONTRIBUTIONS

E.B. and G.B. wrote the manuscript with contributions from all other authors. E.B., T.H., F.D., P.D., and G.B. designed and drew the figures. All authors read and approved the manuscript for submission.

DATA AVAILABILITY STATEMENT

Does not apply as no new data were generated for this manuscript.

ORCID

Erhard Bremer https://orcid.org/0000-0002-2225-7005
Tamara Hoffmann https://orcid.org/0000-0002-5218-8506
Felix Dempwolff https://orcid.org/0000-0002-7788-8445
Patricia Bedrunka https://orcid.org/0000-0002-9984-5518
Gert Bange https://orcid.org/0000-0002-7826-0932

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