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Phenotypic plasticity: The role of a phosphatase family Rap in the genetic regulation of *Bacilli*

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Abstract

In the last two decades, an increasing number of bacterial species have been recognized that are able to generate a phenotypically diverse population that shares an identical genotype. This ability is dependent on a complex genetic regulatory network that includes cellular and environmental signals, as well as stochastic elements. Among *Bacilli*, a broadly distributed family of Rap (Response-regulator aspartyl phosphate) phosphatases is known to modulate the function of the main phenotypic heterogeneity regulators by controlling their phosphorylation. Even more, their related extracellular Phr (Phosphatase regulator) peptides function as signals, creating a cell–cell communication network that regulates the phenotypic development of the entire population. In this review, we examine the role that the Rap phosphatases and their Phr peptides play in the regulation of *Bacillus subtilis* phenotypic differentiation, and in other members of the *Bacillus* genus. We also highlight the contribution of these regulatory elements to the fitness of bacterial cells and mobile genetic elements, for example, prophages and conjugative vectors.

KEYWORDS
Bacillus, biofilm, differentiation, phenotypic heterogeneity, Rap phosphatase

1 | INTRODUCTION

Bacteria in natural settings are constantly exposed to changing environmental conditions, and they must adapt to those changes to survive. Developing a phenotypically heterogeneous population is a strategy that bacteria utilize to increase their environmental fitness, and as a survival mechanism (Smits et al., 2007). This is due to the benefit for the population as a whole driven by cell-level phenotypic differences. Phenotypic heterogeneity can allow specific cells to survive sudden environmental changes that kill other members of the population. It can also lead to division of labor between individuals, which can increase the population's growth rate and facilitate the development of new biological functions (Ackermann, 2015; Davis & Isberg, 2016; West & Cooper, 2016; Zhang et al., 2016). In the last decade, the study of phenotypic heterogeneity among microbial populations and communities has become a major research focus, and new techniques and models are being generated to explore this facet of microbiology (Ackermann, 2015; Claessen et al., 2014; Jo et al., 2022; Shank, 2018).

Bacillus subtilis is a Gram-positive non-pathogenic bacterium that has been studied for over a century in a wide range of topics (Kovács, 2019), and has become a model organism for the study of bacterial differentiation, including community movement on semi-solid agar surfaces, swarming and sliding (Grau et al., 2015; Hölscher & Kovács, 2017; Kearns, 2010), sporulation (Errington, 2003; Khanna et al., 2020; Tan & Ramamurthi, 2014), and biofilm formation (Arnaouteli

et al., 2021; Kovács & Dragoš, 2019; Mhatre et al., 2014). An interesting characteristic of *B. subtilis*, both under planktonic and biofilm conditions, is that its cells divide into discrete subpopulations, each with a different phenotype although all still possessing the same genotype (Kovács & Kuipers, 2013; Veening et al., 2008). This phenotypic differentiation leads to division of labor or bet hedging, providing an important ecological advantage to this bacterium (de Jong et al., 2011; Dragoš et al., 2018; Jautzus et al., 2022; Van Gestel et al., 2015). In the following sections, we address the role that the family of Rap phosphatases and their Phr peptides play in the regulation of *B. subtilis* phenotypic differentiation among *Bacilli*, their mechanism of action and structural functionality, as well as the ecological and genetic reasons that may explain their wide distribution in this genus.

2 | A FAMILY OF REGULATORY PHOSPHATASES

The genetic network of *B. subtilis* contains multiple cross-talk points between the activities of several global regulators that guarantees that the population differentiates accordingly to its environmental conditions (López & Kolter, 2010). Furthermore, *B. subtilis* possesses a family of cell–cell communication Rap phosphatases that fine-tunes this intertwined genetic network (Perego, 2013).

The Rap phosphatases are conserved proteins (>25% of sequence identity) of ca. 380 amino acids that are able to hinder the phosphorylation of global regulators and therefore influencing the expression of hundreds of genes (Perego, 2013; Pottathil & Lazazzera, 2003). Early studies showed that the primary function of these proteins is to directly dephosphorylate their target regulators. The exception is the regulation of Spo0A, where the cognate Rap phosphatases act on upstream members of the phosphorelay, such as Spo0F~P (Perego et al., 1994; Veening et al., 2005). Some Rap phosphatases can also bind to their target transcriptional regulators, forming a complex that can no longer adhere to DNA (Bongiorni et al., 2005; Core & Perego, 2003). Shortly after the discovery of the first Rap phosphatases (RapA and RapB), it was recognized that a small gene directly following *rapA* was involved in RapA regulation: its expression results in the production of a five-amino acid peptide that binds and inhibits the activity of RapA, and thus was called PhrA, phosphatase regulator A (Perego & Hoch, 1996). It has been early realized that Phr peptides have important function in affecting different developmental pathways of *B. subtilis* (Lazazzera et al., 1999; Solomon et al., 1995, 1996).

Subsequent studies have later revealed a wide variety of *rap* genes in the genome of *B. subtilis*, most of which are followed (and slightly overlapped) by *phr* genes that code for small proteins of ca. 40 amino acids known as Phr pro-peptides. The *rap-phr* gene pairs are often recognized as cassettes, and the production of their respective proteins is translationally coupled (Even-Tov, Omer Bendori, Pollak, et al., 2016; Pedreira et al., 2022; Reizer et al., 1997). Once produced, the Rap phosphatases can immediately exert their regulatory function, either by dephosphorylating or preventing the DNA-binding of their target transcriptional regulator (Figure 1). The pro-peptides encoded by the *phr* genes follow a more complicated path to become active. Phr pro-peptides contain export signal sequences in their N-terminal portion, followed by signal peptide cleavage domains and hydrophilic C-terminal domains (Stephenson et al., 2003). The Phr pro-peptides are mobilized to the cell membrane, where they are processed by peptidases that produce 5–6 amino acid Phr peptides in the extracellular space. The mature Phr peptides, upon reaching threshold concentrations, are imported back into the cell by the Opp

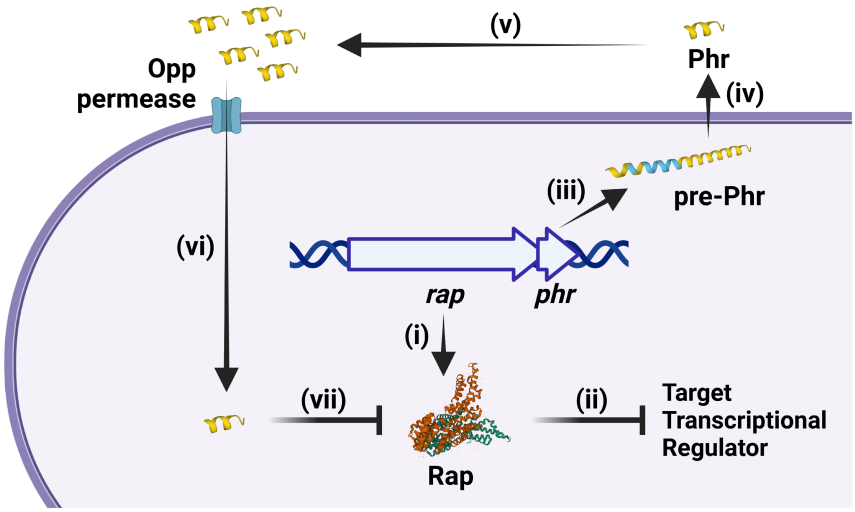

FIGURE 1 General regulatory mechanism of Rap-Phr pairs. (i) the Rap protein is produced, and (ii) carries its regulatory role intracellularly; meanwhile, (iii) pre-Phr proteins are produced, (iv) processed and exported out of the cell as Phr peptides; (v) upon reaching threshold concentrations, (vi) mature Phr peptides can be imported into the cell via the Opp permease, and (vii) inhibit its cognate Rap protein. Rap proteins are visualized using the RapF crystal structure (PDB doi: 10.2210/pdb4l9E/pdb), the red and green colors indicate two units of a protein dimer. The prePhr is depicted using the structure based on AlphaFold as displayed in SubtiWiki (Pedreira et al., 2022). The figure was prepared on BioRender.com.

oligopeptide permease (Lazazzera et al., 1999; LeDeaux et al., 2006; Solomon et al., 1995). Once inside the cell, Phr peptides can finally directly bind to their respective cognate Rap phosphatase and induce a conformational change that blocks Rap activity (Gallego del Sol & Marina, 2013; Neiditch et al., 2017; Perego, 2013; Pottathil & Lazazzera, 2003).

3 | MASTER REGULATORS OF PHENOTYPIC DIFFERENTIATION IN *B. SUBTILIS*

In *B. subtilis*, the response regulators Spo0A, ComA, and DegU are recognized as the master switches that control the development of population heterogeneity (Kovács, 2016). The activity of these three heterogeneity modulators depends on the ratios of the respective proteins in their non-phosphorylated and phosphorylated states (phosphorylated regulators are henceforth indicated with ~P). In general, the phosphorylation state affects the regulator's affinity for the promotor regions of the genes that they regulate (Kobayashi, 2007; Kovács, 2016). Delicate modulation of these ratios allows *B. subtilis* to develop a heterogeneous population, where cells adapt to small environmental differences (micro-niches) within the population, especially in the spatial structure of biofilms (Arnaouteli et al., 2021; López et al., 2009; Shank & Kolter, 2011).

Spo0A is a transcriptional regulator that controls the expression of hundreds of genes and operons in *B. subtilis* important for biofilm matrix production and the generation of spores (Errington, 2003). Spo0A is phosphorylated via a phosphorelay that can be initiated by any of five known independent histidine kinases (KinA, KinB, KinC, KinD, and KinE; Arnaouteli et al., 2021; Jiang et al., 2000). Once activated by their cognate signals, the Kin kinases phosphorylate the response regulator Spo0F, which in turn transfers the phosphoryl group to a secondary response regulator, Spo0B, which finally phosphorylates Spo0A. The amount of Spo0A~P in the cells determines which of its target genes are expressed, toxins are expressed at low Spo0A~P and sporulation is activated at high Spo0A~P level in the cells (Fujita et al., 2005; Kovács, 2016).

The *comXQPA* genes encode a quorum sensing (QS) system, including the global regulator, ComA protein (López et al., 2009). QS is a common cell communication strategy that relies on the production and detection of extracellular autoinducer signaling molecules by cells of the same species (Waters & Bassler, 2005; Whiteley et al., 2017). The ComA QS system comprises the ComX peptide as its autoinducer and the membrane-localized ComP histidine kinase as the sensor. The extracellular ComX signal activates ComP, leading to autophosphorylation and transfer of the phosphate to ComA (Grossman, 1995; Mielich-Süss & Lopez, 2015). Once phosphorylated, ComA controls the production of surfactin (an important surfactant lipopeptide), and the development of competence in *B. subtilis* cells (Nakano et al., 1991).

The third major master switch of population heterogeneity in *B. subtilis*, DegU is the response regulator of the DegS/U two component system. DegS is a cytoplasmic sensor histidine kinase that directly phosphorylates DegU. The DegU regulon is extensive and includes genes associated with motility (e.g., flagellum production) and biofilm formation (e.g., the hydrophobin protein BslA and exoenzymes needed for substrate degradation; Murray et al., 2009). Non-phosphorylated DegU activates competence development. Depending on the relative amount of DegU~P, different sets of genes are transcribed that provide a smooth transition from surface spreading to settlement during biofilm development (Dahl et al., 1991; Kobayashi, 2007; Kovács, 2016).

4 | REGULATORY FUNCTION AND MECHANISMS OF THE RAP-PHR CASSETTES

The regulatory function and mechanism of action of the Rap phosphatases and Phr peptides has been intensively studied. The known Rap phosphatases have been studied in diverse strains, finding an apparent redundancy in their function: most Rap phosphatases act upon Spo0F~P, ComA~P, or both (Bischofs et al., 2009; Grossman, 1995), in addition to specific Rap phosphatases influencing DegU (Ogura et al., 2002). Furthermore, these investigations have revealed that certain Rap-Phr cassettes are encoded on plasmids, and that these regulatory modulators are a common feature of other members of the *Bacillus* genus (Boguslawski et al., 2015; Cardoso et al., 2019; Even-Tov, Omer Bendori, Pollak, et al., 2016; Koetje et al., 2003; Yang et al., 2015). Table 1 presents the known function of those Rap phosphatases that have been studied or reported independently, along with their possible action mechanism.

All known Rap phosphatases share a high sequence homology; however, they regulate structurally distinct targets (Neiditch et al., 2017; Pottathil & Lazazzera, 2003). Initial structural predictions of Rap phosphatases based on amino acid sequence suggested a two-domain architecture consisting of an N-terminal 3-helix bundle domain connected to a tetratricopeptide repeat (TPR) domain. This structure is strikingly different from other known bacterial phosphatases (Neiditch et al., 2017; Parashar et al., 2011; Parashar, Jeffrey, et al., 2013). TPR domains consist of 3–16 repeats of a degenerate 34 amino acid sequence motif, these repeats create a right-handed superhelix structure with an internal ligand-binding concave surface. TPR domains are known to function as protein–protein interaction domains (Blatch & Lässle, 1999). Rap proteins appear to possess 6 canonical TPR motifs distributed along most of their length, with a further non-canonical TPR motif separating TPR5 and TPR6 (Parashar et al., 2011; Perego, 2001). Parashar et al. found that Rap proteins undergo a major conformational change in their N-terminal domain when complexed with their target proteins: the N-terminal 3-helix bundle is flipped and merged with the existing C-terminal TPR domains (Parashar et al., 2011). Further comparison of the crystal structures of RapI, RapH, and RapF (these last two in complex with their target proteins) revealed that these conformational changes can generate

TABLE 1 Known Rap-Phr proteins and their regulatory role in *Bacillus subtilis*.

Rap protein	Phr peptide	Gene cluster localization	Target regulator and action mechanism	References
RapA	PhrA	Chromosome	Dephosphorylation of Spo0F~P	(Diaz et al., 2012; Ishikawa et al., 2002)
RapB	PhrC	Chromosome	Dephosphorylation of Spo0F~P	(Perego, 1997; Pottathil & Lazazzera, 2003; Tzeng et al., 1998)
RapC	PhrC	Chromosome	Blocks binding of ComA to DNA	(Core & Perego, 2003; Lazazzera et al., 1999)
RapD	Unknown	Chromosome	Inhibition of ComA activity	(Ogura and Fujita, 2007)
RapE	PhrE	Chromosome	Dephosphorylation of Spo0F~P	(Jiang et al., 2000; Perego, 2001)
RapF	PhrF	Chromosome	Blocks binding of ComA to DNA	(Auchtung et al., 2005; Bongiorni et al., 2005)
RapG	PhrG	Chromosome	Blocks binding of DegU to DNA. Possible inhibition of ComA activity	(Hayashi et al., 2006; Ogura et al., 2002)
RapH	PhrH	Chromosome	Dephosphorylation of Spo0F~P. Blocks binding of ComA to DNA	(Gallego del Sol & Marina, 2013; Mirouze et al., 2011; Parashar et al., 2011; Smits et al., 2007)
RapI	PhrI	Chromosome	Dephosphorylation of Spo0F~P. Involved in the regulation of mobile genetic elements	(Auchtung et al., 2005, 2007; Gallego del Sol & Marina, 2013; Parashar, Jeffrey, & Neiditch, 2013)
RapJ	PhrC	Chromosome	Possible dephosphorylation of Spo0F~P	(Parashar et al., 2011; Parashar, Jeffrey, et al., 2013)
RapK	PhrK	Chromosome	Inhibition of ComA activity	(Auchtung et al., 2006; Gallego del Sol & Marina, 2013)
RapLS20	Unknown	Plasmid	Possible dephosphorylation of Spo0F~P Regulatory effect on sporulation. Involved in the regulation of mobile genetic elements	(Meijer et al., 2021; Mori et al., 2021; Rösch et al., 2014; Singh et al., 2013)
RapP	PhrP (RapP is resistant to PhrP)	Plasmid	Dephosphorylation of Spo0F~P. Inhibition of ComA activity	(Omer Bendori et al., 2015; Parashar, Konkol, et al., 2013)
RapQ	PhrQ	Plasmid	Inhibition of ComA activity. Possible dephosphorylation of Spo0F~P. Originally identified in *Bacillus amyloliquefaciens*.	(Yang et al., 2015)
Rap40	Phr40	Plasmid	Unknown	(Meijer et al., 1998)
Rap50	Phr50	Plasmid	Unknown	(Meijer et al., 1998)
Rap60	Phr60	Plasmid	Dephosphorylation of Spo0F~P. Inhibition of ComA activity	(Boguslawski et al., 2015; Koetje et al., 2003; Meijer et al., 1998)

different interacting surfaces that block their target's active site (in the case of Spo0F), or DNA-binding domain (in the case of ComA; Baker & Neiditch, 2011; Parashar et al., 2011; Parashar, Jeffrey, et al., 2013).

The regulatory mechanism of the Phr peptides has also been structurally studied. Binding of Rap proteins to their cognate Phr peptides is mediated by their C-terminal TPR domains, and causes a pronounced rotation of the N-terminal 3-helix bundle; this creates two helix-turn-helix structures that pack against the existing C-terminal TPR domain. This rearrangement generates a compression along the whole TPR superhelical axis, which causes the loss of the ligand-binding concave surface normally present in Rap proteins. Furthermore, the Phr peptides can interact with the residues of multiple TPR repeats (up to six, in the case of RapF-PhrF complexes), leading to intramolecular interactions that stabilize the "closed" conformation of the Rap protein (Gallego del Sol & Marina, 2013; Parashar, Jeffrey, et al., 2013). These multi-TPR motif interactions confer a high specificity to Rap-Phr binding, with some Phr residues determining protein anchoring and orientation, and others mediating the interaction with the residues of the Rap protein. Gallego del Sol and Marina (2013) demonstrated that specific residues of RapF are required to bind its PhrF inhibitor, and that these residues are independent from the ability of RapF to bind to its target regulator ComA. The conservation of similar residues among Rap proteins, and additional experimental evidence from previous studies (Diaz et al., 2012) suggest that this is a common Phr-binding mechanism for all Rap proteins.

Interestingly, a few known *rap* genes lack the concomitant gene for a specialized Phr peptide, but can be regulated by Phr peptides produced by other Rap-Phr cassettes (see SubtiWiki http://subtiwiki.uni-goettingen.de; Pedreira et al., 2022). This is the case of RapB, which lacks a specialized Phr but is regulated by PhrC in vitro (Even-Tov, Omer Bendori, Pollak, et al., 2016; Perego, 1997). Another example is *rapJ*, which is not followed by a *phr* gene. RapJ plays its regulatory role by dephosphorylating Spo0F~P, and it binds to PhrC, forming a complex that is no longer able to interact with Spo0F~P (Even-Tov, Omer Bendori, Pollak, et al., 2016; Parashar, Jeffrey,

et al., 2013). Moreover, at least one Rap protein is known to be insensitive to regulation by its cognate Phr peptide. The *rapP-phrP* cassette is encoded on the pBS32 plasmid present in the undomesticated strain of *B. subtilis* NCIB 3610. RapP regulates biofilm formation, sporulation, and competence development by directly dephosphorylating Spo0F~P (McLoon et al., 2011; Parashar, Konkol, et al., 2013), and by a ComA-dependent mechanism (Omer Bendori et al., 2015). However, RapP is not inhibited by PhrP, either when PhrP is overexpressed in vivo, or tested in vitro with exogenously added peptides derived from the C-terminal sequence of *phrP* (Omer Bendori et al., 2015; Parashar, Konkol, et al., 2013). Conspicuously, RapP of *B. subtilis* NCIB 3610 shows an asparagine-to-threonine mutation at position 236 that is not present in the corresponding *rapP* alleles of other *Bacillus* strains. Omer Bendori et al. (2015) showed that this single amino acid substitution is responsible for the observed resistance of RapP to inhibition by PhrP, and that this inhibition could be restored by repairing the N236T mutation. Similarly, the plasmid encoded Rap63-Phr63 and Rap8-Phr8 synergistically modulate sporulation and biofilm formation of *B. thuringiensis* (Cardoso et al., 2020; Fazion et al., 2018).

The structural insights from these studies suggest that there is a delicate balance between peptide-recognition specificity and regulatory plasticity in the Rap-Phr family. Studies that use various synthetic Phr peptides to investigate Rap-Phr interactions have found that although usually one peptide shows strong affinity for a given Rap protein (normally the one coded in the same *rap-phr* cassette), other Phr peptides also show a partial ability to regulate the same Rap protein in vitro and in vivo (Diaz et al., 2012; Even-Tov, Omer Bendori, Pollak, et al., 2016; Gallego del Sol & Marina, 2013; Parashar et al., 2011; Perego, 1997). These alternative Phr are usually synthetized comprising the last 5 or 6 residues of the C-terminal end of Phr pro-peptides. These experiments highlight the orthogonality of Rap-Phr systems, especially within the same genome (Even-Tov, Omer Bendori, Pollak, et al., 2016). However, it is important to consider that these results have been observed using artificial laboratory conditions, and in particular, in vitro experiments include the testing of a very limited number of peptides at a time. In natural settings, a complex network of Phr peptide cross-talk and co-regulation might exist among the populations of *Bacilli* to modulate the function of Rap phosphatases.

5 | DISTRIBUTION AND DIVERSITY OF THE RAP-PHR FAMILY IN THE *BACILLUS* GENUS

The Rap-Phr family of regulatory proteins is highly diversified and widespread in the *Bacillus* genus. Rap homologs have been found in the genomes of *B. subtilis*, *Bacillus amyloliquefaciens*, *Bacillus pumilus*, *Bacillus anthracis*, *Bacillus cereus*, *Bacillus halodurans*, *Bacillus stearothermophilus*, and *Bacillus clausii* (Cardoso et al., 2019; Even-Tov, Omer Bendori, Pollak, et al., 2016; Perego, 2001; Takami et al., 2000; Yang et al., 2015). Also, one of these Rap-Phr cassettes, RapQ-PhrQ from *B. amyloliquefaciens* has been studied heterologously in *B. subtilis*, and was both functional and comparable to the native Rap phosphatases of the host (Yang et al., 2015). These reports support the idea that the Rap proteins and their Phr peptides play a common regulatory role in the entire *Bacillus* genus.

Interestingly, *Bacillus* species encode 2–20 of the Rap-Phr cassettes in their genomes. The first study to identify RapA as a Spo0F phosphatase already established RapA and RapB as members of a protein–aspartate phosphatase family with multiple members within the same organism: *B. subtilis* strain JH642 (Perego et al., 1994). Subsequent studies have identified further members of this phosphatase family in diverse *B. subtilis* strains (see Table 1). In 2016, Even-Tov et al. compared over 400 *Bacillus* genomes, searching for Rap homologs based on their conserved N-terminal 3-helix bundle and C-terminal TPR domain structure. 2500 *rap* homologs were identified among the studied genomes. *B. subtilis* strains have, on average, 11 *rap* genes per strain, while *B. cereus* strains usually possess around 6 of these phosphatases (Even-Tov, Omer Bendori, Pollak, et al., 2016). Interestingly, Rap homologs are clearly clustered to two groups, corresponding to the division between the *B. subtilis* and *B. cereus* species groups, which suggests the divergence of *rap* genes in each species complex occurred after their evolutionary separation (Even-Tov, Omer Bendori, Pollak, et al., 2016).

How has the Rap-Phr family achieved such a widespread presence and diversity among *Bacilli*? There are two main factors that can be considered when answering this question. First, bacteria commonly pass on genes among sibling cells or cells from closely related species. This ability, known as horizontal gene transfer (HGT), is an efficient mechanism for individual organisms to acquire genes, regardless of functionality (Ochman et al., 2000; Soucy et al., 2015). HGT of Rap-Phr cassettes is heightened due to the fact that many *rap-phr* genes are encoded within mobile genetic elements (Bongiorni et al., 2006; Butala & Dragoš, 2022; Meijer et al., 2021; Mori et al., 2021; Parashar, Konkol, et al., 2013; Rösch et al., 2014; Singh et al., 2013). Importantly, Rap-Phr can also control mobile elements, as RapI-PhrI sensory system activates gene expression within the ICEBs1 mobile genetic element, therefore contributing to excision and transfer (Auchtung et al., 2005). In addition, the genes related to natural competence for the uptake of DNA from the environment are widely conserved in *Bacilli* (Kovács et al., 2009). Even-Tov et al. estimated that up to 75% of Rap-Phr cassettes may be mobile (i.e., coded on plasmids, prophages, or integrative and conjugative elements), based on a GC-content comparison with their host strain (Even-Tov, Omer Bendori, Pollak, et al., 2016). Furthermore, some Rap-Phr cassettes are able to regulate the mobility of the genetic element that contains them, be them plasmids (Meijer et al., 2021; Mori et al., 2021; Singh et al., 2013), or transposons (Auchtung et al., 2005, 2007). These features could then favor a rapid expansion of Rap-Phr cassettes through HGT among *Bacilli*. Similarly, experimental selection for spores of *B. subtilis* increases the copy number of a cryptic prophage, phi3T, that carries Rap and Phr proteins (Dragoš, Priyadarshini, et al., 2021; Martin et al., 2017). Interestingly, certain prophages, like SPβ, that are similar to phi3T do not carry such *rap* gene, but encode a biosynthetic gene cluster for a

bacteriocin, sublancin that presumably benefit the fitness of the host bacterium (Butala & Dragoš, 2022; Dragoš, Andersen, et al., 2021; Dragoš, Priyadarshini, et al., 2021; Floccari & Dragoš, 2023). The Rap protein coded within the phi3T prophage has been also hypothesized to contribute to phage fitness (Bernard et al., 2021). Further, genome analysis combined with targeted experimental validation revealed that diversification of the autoinducer Phr peptides might be driven by promiscuous duplication events followed by adjustment of the Phr peptide in accordance with the respective evolutionary change of its cognate Rap phosphatase (Even-Tov, Omer Bendori, Pollak, et al., 2016).

A second factor that can help explain the diversity of the Rap-Phr family is functional diversification through social selection. Experimental analyses and modeling suggest that acquisition of additional Rap-Phr system is facilitated by a facultative social cheating mechanism in *B. subtilis* (Even-Tov, Omer Bendori, Valastyan, et al., 2016). At low frequency, a strain harboring an extra Rap-Phr system acts as a cheater (i.e., it exploits the public good produced by the corresponding wild-type), while at high frequency, it adheres to cooperation without fitness loss. Such social selection in combination with HGT ensures the diversification and maintenance of multiple copies of Rap-Phr systems in *Bacilli*. Interestingly, the advantage of having multiple quorum sensing systems, that is, ComX-dependent activation of ComP/ComA system and Phr-affected Rap phosphatases, is not only apparent in *B. subtilis*, but also present in other bacteria, for example, in *Vibrio harveyi* (Even-Tov, Omer Bendori, Valastyan, et al., 2016). The ComX and Phr-based cell–cell communication systems work in concert to adjust developmental pathways of *B. subtilis* (Lazazzera et al., 1999; Solomon et al., 1995). Importantly, the signaling molecules of these alternative quorum sensing systems might diffuse distinctly within the cells' environment, therefore, defining the potential range of communication (Van Gestel et al., 2021).

6 | SOCIAL REGULATION OF *B. SUBTILIS* PHENOTYPIC ADAPTABILITY THROUGH RAP PHOSPHATASES

Rap phosphatases fine-tune the sociomicrobiology of *B. subtilis* by modulating the activity of the master regulators Spo0A, ComA, and DegU and therefore their regulons (Figure 2). This fine-tuning occurs at different levels: first, the Rap phosphatases themselves that may appear at first sight to have directly redundant regulatory roles, are expressed under different conditions. Various regulators have been

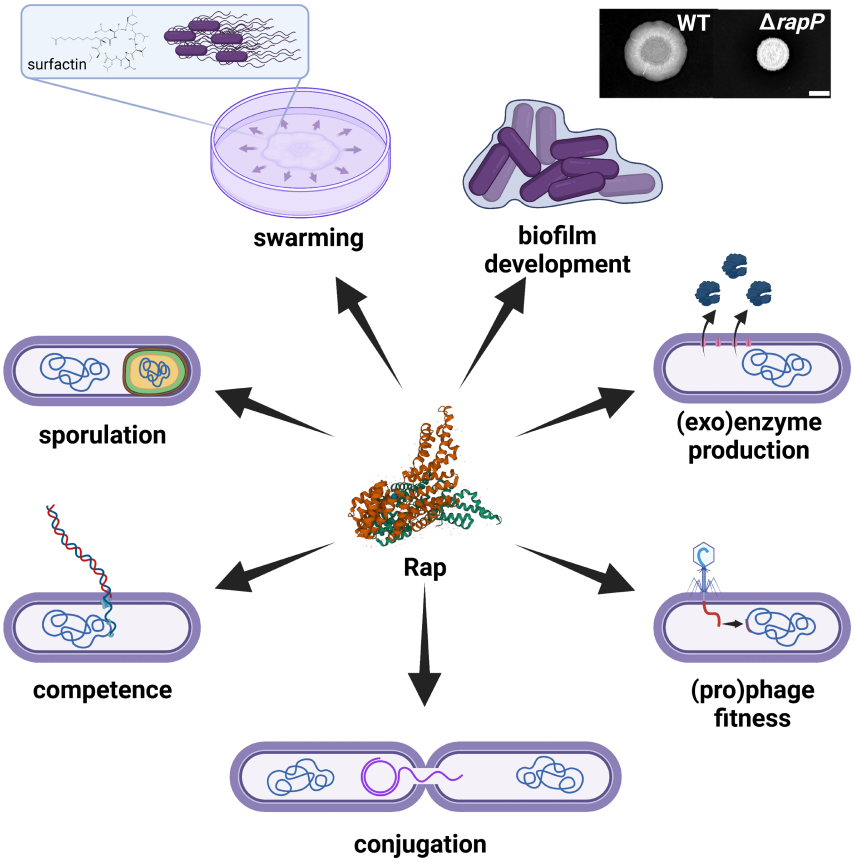

FIGURE 2 Known cellular functions regulated by Rap phosphatases in *Bacillus subtilis*. The central protein structure shows the conserved architecture of Rap phosphatases (RapF crystal structure, PDB doi: 10.2210/pdb4I9E/pdb). The red and green colors indicate two units of a protein dimer. For detailed description of the Rap protein domains involved in interaction with Phr or target regulators, see Neiditch et al. (2017). The biofilm colony images of wild-type (WT) and Δ*rapP* mutant are taken from Nordgaard et al. (2021), scale bar represents 5 mm. The figure was prepared on BioRender.com.

identified to influence the expression of *rap-phr* genes (Table 2 and Figure 3). This leads to regulatory differences among Rap proteins with the same target. As an example, both RapA and RapB dephosphorylate Spo0F~P, however, *rapA* expression is promoted by QS-dependent ComA, while *rapB* seems to be promoted only by the house-keeping sigma factor σ^A (Comella & Grossman, 2005; Jarmer et al., 2001; Mueller et al., 1992). This difference means that RapB will be produced earlier and more consistently than RapA, leading to differences in the Spo0A/Spo0A~P ratio in the cell population. A second level of fine-tuning is given by the Phr peptides, which may comprise the most diverse family of cell–cell communication autoinducers known to date (Even-Tov, Omer Bendori, Pollak, et al., 2016). Cell–cell communication systems are used to coordinate social behavior that is most effective if a large number of cells participate, such as expression of virulence, or production of a biofilm matrix. The different Rap-Phr pairs have distinct influence on biofilm development of *B. subtilis* and colonization of the roots of the model plant *Arabidopsis thaliana* (Nordgaard et al., 2021). In biofilm settings, the process-export–import regulatory pathway of Phr peptides provides *B. subtilis* cells with the opportunity to detect and integrate further environmental signals into their complex gene regulatory network. Cells in a biofilm live in micro-niches that lead to population heterogeneity (Christensen et al., 2002; López et al., 2009; Martin et al., 2016; Otto et al., 2020), thus, the biofilm subpopulations will secrete different types and amounts of mature Phr peptides to the extracellular milieu. Furthermore, the flexibility of Phr peptides to serve as cell–cell communication signals has been demonstrated by the ability of non-producing cells to detect the Phr signals produced by other cells (Pottathil & Lazazzera, 2003; Veening et al., 2005). Therefore, Phr peptides can form a biofilm-spanning communication network, where each biofilm subpopulation can participate in the developmental process of their neighbors.

TABLE 2 Known sigma factors and transcriptional regulators of *rap-phr* genes in *Bacillus subtilis* based on SubtiWiki (Pedreira et al., 2022).

The *rap-phr* gene	Sigma factor	Regulator
rapA-phrA	σ^A	ComA, CodY, CcpA, Spo0A
rapB	σ^A	
rapC-phrC	σ^A, σ^H	CodY, ComA, CcpA
rapD	σ^A, σ^M, σ^X	RghR
rapE-phrE	σ^A, σ^H	ComA, CodY
rapF-phrF	σ^H	ComA, CcpA
rapG-phrG	σ^A, σ^H	SinR, RhgR, CcpA
rapH-phrH	σ^A	AbrB, ComK, RhgR
rapI-phrI	σ^A, σ^H	ComA
rapK-phrK	σ^H	AbrB, PhoP

Note: References to regulons: σ^A (Jarmer et al., 2001; Mirouze et al., 2011); σ^H (Lazazzera et al., 1999; McQuade et al., 2001); σ^X (Huang and Helmann, 1998); σ^M (Eiamphungporn and Helmann, 2008); ComA (Comella & Grossman, 2005); CodY (Belitsky and Sonenshein, 2013; Molle, Nakaura, et al., 2003); CcpA (Blencke et al., 2003); Spo0A (Molle, Fujita, et al., 2003); RhgR (Hayashi et al., 2006; Ogura and Fujita, 2007); AbrB (Chumsakul et al., 2011); ComK (Berka et al., 2002; Hamoen et al., 2002; Ogura et al., 2002); SinR (Chu et al., 2006); PhoP (Salzberg et al., 2015).

7 | RAP PHOSPHATASES AFFECT STRAIN FITNESS THROUGH TIMING OF SPORULATION

B. subtilis strains have been isolated from diverse environment, including soil, animals, plants and aquatic habitats (Connor et al., 2010; Earl et al., 2008; Kovács, 2019). Interestingly, although *B. subtilis* strains commonly show conservation among their main population heterogeneity regulators (Spo0A, ComA, DegU; Earl et al., 2008; Serra et al., 2014), they show high variation among their Rap-Phr cassette content (Even-Tov, Omer Bendori, Pollak, et al., 2016). Rap phosphatases determine the phenotypic memory of *B. subtilis* spores, the timing of spore formation and germination speed: the earlier the spores are formed, the faster these spores germinate driven by higher level of alanine dehydrogenase (i.e., high-quality spores), while delayed establishment of spores lead to higher number of spores in the population with reduced revival ability (i.e., high-quantity spores; Mutlu et al., 2018). Overexpression of *rapA* gene slows sporulation, vegetative cells have more time to grow and multiply, which increases the spores yield. However, this gives rise to a lower fraction of spores germinating (Mutlu et al., 2018). The differences in the number and the diversity of the Rap-Phr family play an important role for the environmental adaptability of specific strains of *B. subtilis* by allowing them to fine-tune their metabolism to different ecological niches. For example, strains isolated from the digestive tract of animals can show differences in the timing of sporulation initiation influenced by Rap-Phr cassette variation (Serra et al., 2014). This variation effectively serves as an adaptation that allows them to sporulate at optimum rates according to the ecological niche in which they live (Serra et al., 2014). Ultimately, these differences in the amount of Rap phosphatases and the timing of sporulation eventually influence the quality-yield spore tradeoff in natural isolates (Mutlu et al., 2020).

Motivated by these observations related to the divergence in *rap-phr* modules and their influence on timing of sporulation, Gallegos-Monterrosa and colleagues constructed all possible combinations of single and double *rap-phr* deletions in *B. subtilis* NCIB 3610 and tested the fitness of these strains in a selection experiment (Gallegos-Monterrosa et al., 2021). A mixture of the single and double mutants along with the wild-type strain was cultivated either as biofilms or as planktonic cultures for 2- or 5-days, and spores were selected for re-inoculation. The initial hypothesis was that longer cultivation time (5 days instead of 2 days) allows enrichment of *rap* mutants that permit longer growth phase before cells are committed to sporulation. After 9 cycles of spore selection, the abundance of strains in each culture condition was determined based on genome integrated, strain-specific barcodes. This approach revealed that a shorter incubation

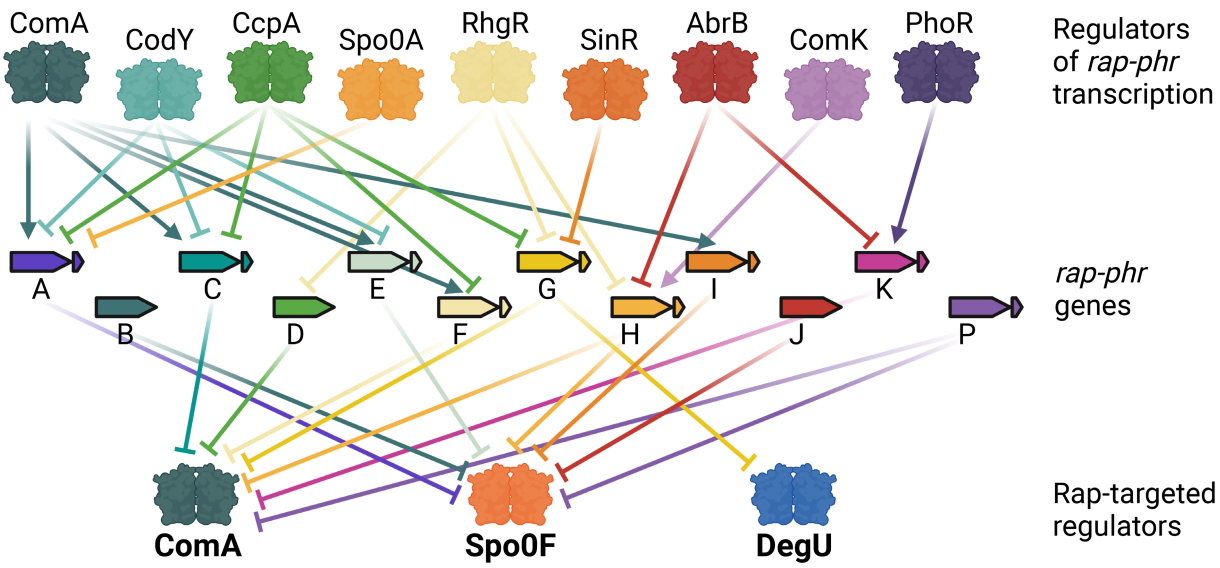

FIGURE 3 Complexity of Rap-Phr network. Top row depicts the regulators that influence the expression of *rap-phr* genes. The activities of proteins ComA, Spo0F, and DegU are hindered by corresponding Rap proteins (bellow). For details, see also Tables 1 and 2. The figure was prepared on BioRender.com.

time selects for higher diversity of mutant strains, while longer cultivation selects for specific combination of *rap-phr* deletions (Gallegos-Monterrosa et al., 2021). Additionally, fitness assays using the mutants that were selected under these conditions, for example, derivative carrying a *rapB* or *rapH* deletion in addition to other *rap-phr* mutation, demonstrated the competitiveness of these strains compared with the wild-type strain. As these evolution experiments included spore selection, the cryptic phi3T prophage and its encoded Rap protein could have potentially influenced the selection dynamics (see above; Dragoš, Priyadarshini, et al., 2021; Gallegos-Monterrosa et al., 2021). Nevertheless, this experimental approach highlighted the complexity of how the different Rap-Phr systems contribute to fitness of *B. subtilis*, although the exact molecular details are yet to be discovered.

8 | CONCLUDING REMARKS

The efficiency of bacterial adaptation depends on the regulatory pathways that enable the cell to sense and respond to the external environment that encompasses both abiotic and biotic factors. Biotic factors include the bacterial population itself and its density. *Bacilli* evolved to integrate population density using the Rap-Phr cell–cell communication pathways that eventually diverged to modulate distinct, but partly overlapping, regulatory systems in the bacteria. Systemic dissection of the Rap-Phr systems (i.e., single- and multiple-deletions of the casettes) in different *Bacillus* species under diverse conditions, including their natural environments, will reveal their impact on the ecology of this group of microorganisms.

AUTHOR CONTRIBUTIONS

Ramses Gallegos-Monterrosa: Writing—original draft. Ákos T. Kovács: Writing—original draft; Funding acquisition.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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