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Modulation of the ComA-Dependent Quorum Response in *Bacillus subtilis* by Multiple Rap Proteins and Phr Peptides†

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In *Bacillus subtilis*, extracellular peptide signaling regulates several biological processes. Secreted Phr signaling peptides are imported into the cell and act intracellularly to antagonize the activity of regulators known as Rap proteins. *B. subtilis* encodes several Rap proteins and Phr peptides, and the processes regulated by many of these Rap proteins and Phr peptides are unknown. We used DNA microarrays to characterize the roles that several *rap-phr* signaling modules play in regulating gene expression. We found that *rapK-phrK* regulates the expression of a number of genes activated by the response regulator ComA. ComA activates expression of genes involved in competence development and the production of several secreted products. Two Phr peptides, PhrC and PhrF, were previously known to stimulate the activity of ComA. We assayed the roles that PhrC, PhrF, and PhrK play in regulating gene expression and found that these three peptides stimulate ComA-dependent gene expression to different levels and are all required for full expression of genes activated by ComA. The involvement of multiple Rap proteins and Phr peptides allows multiple physiological cues to be integrated into a regulatory network that modulates the timing and magnitude of the ComA response.

Many bacteria use extracellular signaling molecules to coordinate a variety of biological processes (reviewed in references 43 and 80). Extracellular signaling molecules can provide information about the density of a population of cells. This regulatory strategy, often called quorum sensing, allows processes to be initiated or inhibited once a certain cell population density is achieved (43, 80). In addition, extracellular signaling molecules may be used to sense the amount of diffusion and mixing occurring within a population of cells; this strategy may allow cells to increase production of extracellular enzymes and secreted products under conditions in which these enzymes are less likely to diffuse away and more likely to provide benefits to the producing cells (63).

In the gram-positive bacterium *Bacillus subtilis*, several processes are known to be regulated by extracellular peptide signaling, including the initiation of genetic competence (the ability to incorporate exogenous DNA from the environment) (41, 70), sporulation (59, 70), production of degradative enzymes (9, 52) and exopolysaccharides (9, 77), and antibiotic synthesis (9, 41, 70). Three types of secreted peptide signaling molecules have been identified: a modified 5- to 10-amino-acid peptide, ComX, that interacts extracellularly with its receptor (41, 62, 76); lantibiotic peptides, such as subtilin, which interact extracellularly with their receptors (reviewed in reference 73); and unmodified pentapeptides, known as Phr peptides, that are internalized to inhibit the activity of their target proteins, known as Rap proteins (reviewed in references 31 and 56).

B. subtilis encodes a family of 8 Phr peptides (PhrA, PhrC, PhrE, PhrF, PhrG, PhrH, PhrI, and PhrK) and a family of 11 Rap proteins (RapA to RapK) (24, 30). Each Phr peptide is

encoded in an operon with a Rap protein (30), and each characterized Phr inhibits the activity of its cotranscribed Rap (6, 27, 52, 59, 70). The PhrC peptide (also known as competence- and sporulation-stimulating factor [70]) also inhibits the activity of an unpaired Rap protein, RapB (55). It is possible that the other unpaired Rap proteins are also inhibited by noncognate Phr peptides.

In addition to expression from the upstream *rap* promoter (32, 59), most *phr* genes are also expressed from a promoter upstream of *phr* that is recognized by RNA polymerase containing the alternative sigma factor, σ^H (32, 42, 47). This regulation by σ^H causes the level of each *phr* gene to increase as cells transition from exponential growth to stationary phase (32, 42).

The primary *phr* gene products are pre-Phr peptides that are 38 to 57 amino acids in length. Pre-Phr peptides are exported and cleaved to form the mature Phr pentapeptides (reviewed in references 31 and 56). The oligopeptide permease (Opp), an ATP-binding cassette (ABC) transporter that imports small peptides (58, 65), transports the Phr peptides into the cell, where they can inhibit the activities of Rap proteins (34, 55).

Several Rap proteins regulate a variety of processes by antagonizing the activities of response regulator proteins, either by stimulating dephosphorylation of the response regulator (RapA, RapB, and RapE) (27, 57, 78) (Table 1) or by binding to the response regulator and interfering with DNA binding (RapC, RapF, and RapG) (6, 10, 52). One Rap protein, RapI, regulates the mobility of the genetic element ICEBsI, possibly by antagonizing the activity of the element-encoded immunity repressor (2). RapG and RapH were recently shown to inhibit expression of some ComA-activated genes, as well as the expression of DegU-activated genes (24). The remaining Rap proteins (RapD, RapJ, and RapK) have not been characterized.

Two Rap proteins, RapC and RapF, inhibit the activity of the response regulator ComA (6, 10, 70). Their cognate peptides, PhrC and PhrF, stimulate ComA's activity by directly

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TABLE 1. Processes regulated by Rap proteins and Phr peptides in *Bacillus subtilis*

Rap protein	Phr peptide	Target(s) of Rap	Mechanism of Rap	Responses regulated by target protein(s)	Reference(s)
RapA	PhrA	Spo0F~P	Stimulates autodephosphorylation	Activates post-exponential-phase gene expression and sporulation indirectly through Spo0A	14, 57, 59
RapB	PhrC	Spo0F~P	Stimulates autodephosphorylation	Activates post-exponential-phase gene expression and sporulation indirectly through Spo0A	14, 55, 57
RapC	PhrC	ComA	Inhibits binding of ComA to DNA	Activates expression of genes involved in production of degradative enzymes, antibiotics, and competence	9, 10, 70
RapD RapE	PhrE	Unknown Spo0F~P	Unknown Stimulates autodephosphorylation	Activates post-exponential-phase gene expression and sporulation indirectly through Spo0A	14, 27
RapF	PhrF	ComA	Inhibits binding of ComA to DNA	Activates expression of genes involved in production of degradative enzymes, antibiotics, and competence	6, 9; this work
RapG	PhrG	DegU, ComA ^a	Inhibits binding of DegU to DNA, unknown	Activate expression of genes involved in competence and production of degradative enzymes and antibiotics	24, 30, 40, 52, 54
RapH	PhrH	ComA ^a , DegU ^a	Unknown	Activate expression of genes involved in competence and production of degradative enzymes and antibiotics	24, 30, 40, 54
RapI	PhrI	Unknown	Unknown	RapI stimulates gene expression, excision, and transfer of ICEBs1	2
RapJ RapK	PhrK	Unknown ComA ^a	Unknown Unknown	Activates expression of genes involved in production of degradative enzymes, antibiotics, and competence	This work

^a Presumed targets of Rap protein.

inhibiting the activities of RapC and RapF (6, 10). ComA, when phosphorylated (50, 64), activates the expression of several genes, including those involved in antibiotic synthesis (9, 50, 51, 82), degradative enzyme production (9, 48), exopolysaccharide production (9), fatty acid metabolism (9), and the initiation of genetic competence (12, 13, 51). ComA receives phosphate from ComP, a membrane-bound receptor histidine kinase that is activated through its interaction with the extracellular signaling peptide ComX (1, 41, 71, 76, 77, 81). Therefore, at least three peptides, i.e., PhrC, PhrF, and ComX, are known to stimulate ComA-dependent gene expression. PhrC and ComX peptide signaling activates ComA-regulated processes when *B. subtilis* cells reach a high population density (41, 70). Signaling through these peptides, as well as the PhrF peptide, may also provide cells with additional physiological information.

In order to more fully understand the roles that Rap proteins and Phr peptides play in modulating ComA-activated processes, we characterized the roles that several Rap proteins and Phr peptides play in regulating ComA-dependent gene expression. We monitored the effects of overproduction of several uncharacterized Rap proteins on global gene expression and found that overproduction of RapK inhibits the expression of genes activated by ComA. Furthermore, deletion of *phrK* resulted in increased expression of ComA-dependent genes. This effect on ComA-dependent gene expression was dependent upon *rapK*, indicating that PhrK stimulates ComA's

activity by inhibiting RapK's activity. We also observed that PhrC, PhrF, and PhrK stimulate ComA-dependent gene expression to different magnitudes and that all three peptides are required for full expression of ComA-dependent genes. Based on these results as well as previously published observations, we conclude that signaling through the PhrC, PhrF, and PhrK peptides integrates multiple physiological cues to modulate the levels and timing of processes regulated by ComA.

MATERIALS AND METHODS

Media. Cells were grown at 37°C in Schaeffer's nutrient broth sporulation medium (DSM) (23) or in S7 minimal salts medium (79) (containing 50 mM instead of 100 mM MOPS [morpholinepropanesulfonic acid]) supplemented with 1% glucose, 0.1% glutamate, tryptophan and phenylalanine (40 µg/ml), and threonine (120 µg/ml) (when necessary) as indicated. LB (66) was used for routine growth of *B. subtilis* and *Escherichia coli*. Antibiotics, when appropriate, were used as follows: ampicillin (100 µg/ml), chloramphenicol (5 µg/ml), neomycin (2.5 µg/ml), spectinomycin (100 µg/ml), erythromycin (0.5 µg/ml) and lincomycin (12.5 µg/ml) together to select for macrolide-lincosamide-streptogramin B resistance, and tetracycline (12.5 µg/ml). Isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma) was used at a final concentration of 1 mM.

Strains and alleles. The strains used in this study are listed in Table 2. All *B. subtilis* strains were derived from the parental strain JH642 (60). The *E. coli* strain used for cloning is an MC1061 derivative with F'(lacI^q) *lacZ*M15 Tn10 (*tet*). Standard techniques were used for cloning and strain construction (23, 66). *Pspank(hy)* (2), *ΔrapC::cat* (70), *ΔphrC::erm* (70), and *amyE::srfA-lacZ*Ω682 (3) were previously described.

For overexpression in *B. subtilis*, the entire *rapC*, *rapF*, *rapH*, *rapJ*, and *rapK* open reading frames (ORFs) were cloned downstream of the IPTG-inducible promoter *Pspank(hy)* (7), a generous gift from D. Rudner (Harvard Medical

TABLE 2. Strains used in this study

Strain name	Genotype ^a
JH642	<i>trpC2 pheA1</i>
CAL7	Δ <i>phrK7::spc</i>
CAL8	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>phrK7::spc</i></i>
CAL9	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>phrC751::erm ΔphrK7::spc</i></i>
CAL10	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>phrF163::cat ΔphrK7::spc</i></i>
CAL11	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>phrC751::erm ΔphrF163::cat ΔphrK7::spc</i></i>
JMA26	<i>amyE::[Pspank(hy)-rapF spc]</i>
JMA27	<i>amyE::[Pspank(hy)-(rapHphrH) spc]</i>
JMA29	<i>amyE::[Pspank(hy)-rapJ spc]</i>
JMA30	<i>amyE::[Pspank(hy)-rapK spc]</i>
JMA47	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>rapC::pJS79 cat</i></i>
JMA48	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>rapK41::cat::erm</i></i>
JMA50	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>rapC::pJS79 cat ΔrapK41::cat::erm</i></i>
JMA51	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>sigH380::cat::spc</i></i>
JMA52	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>oppBCDE585::spc</i></i>
JMA53	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>sigH380::cat::spc ΔrapC::pJS79 cat</i></i>
JMA54	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>oppBCDE585::spc ΔrapC::pJS79 cat</i></i>
JMA55	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>sigH380::cat::spc ΔrapK41::cat::erm</i></i>
JMA56	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>oppBCDE585::spc ΔrapK41::cat::erm</i></i>
JMA57	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>sigH380::cat::spc ΔrapC::pJS79 cat ΔrapK41::cat::erm</i></i>
JMA58	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>oppBCDE585::spc ΔrapC::pJS79 cat ΔrapK41::cat::erm</i></i>
JMA76	<i>amyE::[Pspank(hy)-rapF spc] thrC::[pel-lacZ erm]</i>
JMA77	<i>amyE::[Pspank(hy)-rapK spc] thrC::[pel-lacZ erm]</i>
JMA78	<i>amyE::[Pspank(hy)-rapC spc] thrC::[pel-lacZ erm]</i>
JMA79	<i>amyE::[Pspank(hy) spc] thrC::[pel-lacZ erm]</i>
JMA117	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>rapFphrF312::tet</i></i>
JMA122	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>oppBCDE585::spc ΔrapFphrF312::tet</i></i>
JMA123	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>sigH380::cat::spc ΔrapFphrF312::tet</i></i>
JMA129	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>rapFphrF312::tet ΔrapK41::cat::erm</i></i>
JMA134	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>oppBCDE585::spc ΔrapC::pJS79 cat ΔrapFphrF312::tet</i></i>
JMA135	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>sigH380::cat::spc ΔrapC::pJS79 cat ΔrapFphrF312::tet</i></i>
JMA138	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>oppBCDE585::spc ΔrapFphrF312::tet ΔrapK41::cat::erm</i></i>
JMA139	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>sigH380::cat::spc ΔrapFphrF312::tet ΔrapK41::cat::erm</i></i>
JMA142	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>rapC::pJS79 cat ΔrapFphrF312::tet ΔrapK41::cat::erm</i></i>
JMA144	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>oppBCDE585::spc ΔrapC::pJS79 cat ΔrapFphrF312::tet ΔrapK41::cat::erm</i></i>
JMA149	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>sigH380::cat::spc ΔrapC::pJS79 cat ΔrapFphrF312::tet ΔrapK41::cat::erm</i></i>
JMA163	Δ <i>phrF163::cat</i>
JMA165	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>phrC751::erm</i></i>
JMA166	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>phrF163::cat</i></i>
JMA169	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>phrC751::erm ΔphrF163::cat</i></i>
JMA752	<i>amyE::[srfA-lacZΩ682 neo] Δ<i>phrK7::spc ΔrapK38::cat</i></i>
JMS682	<i>amyE::[srfA-lacZΩ682 neo]</i>
RSM121	Δ <i>phrC751::erm</i>

^a All strains are derived from JH642 and contain *trpC2* and *pheA1* alleles.

School), and integrated into the *amyE* locus by homologous recombination. Cloning of *Pspank(hy)-rapH* was based on the previously published sequence of this ORF (30). Reanalysis of this sequence, based on the newly released sequence published by Hayashi et al. (24), indicates that this construct contains the entire *rapH* and *phrH* ORFs downstream of the *Pspank(hy)* promoter.

rapF-phrF was deleted by replacing (from the start codon) nucleotides +542 of *rapF* to +149 of *phrF* with a tetracycline resistance gene derived from pDG1513 (19). *rapK* was deleted by replacing nucleotides +36 to +980 of *rapK* with the chloramphenicol resistance gene from pGEM-*cat* (83); *cat* was replaced with *erm* by integration of the plasmid pCm::Er (74). *phrF* was deleted by replacing nucleotides +38 to +103 of *phrF* with the chloramphenicol resistance gene from pGEM-*cat*. *phrK* was deleted by replacing nucleotides +64 to +100 of *phrK* with spectinomycin resistance derived from pDL55 (4).

Δ *oppBCDE::spc* (Δ *spo0KBCDE::spc*) was created by replacing the RsrII/ClaI fragment in plasmid pDR9 (65) (contains *oppBCDE*) with the BglII/NdeI fragment containing *spc* from pUS19 (4). Both plasmid and insert DNA was recessed/filled-in by the Klenow fragment of *E. coli* DNA polymerase I prior to ligation. The recombinant plasmid was integrated into the *B. subtilis* chromosome by homologous recombination.

Δ *sigH::cat::spc* (Δ *spo0H::cat::spc*) was created by integrating plasmid pJL62 (35) by homologous recombination into a strain containing the Δ *sigH::cat* (26) mutation.

The *pel-lacZ* promoter fusion was generated by cloning the DNA from nucleotides -371 to +39 of *pel* upstream of the promoterless *lacZ* in the vector pDG793 (18), followed by integration into the *thrC* locus by homologous recombination. A similar fusion at *amyE* was previously described (9).

DNA microarrays. *Pspank(hy)-rapF* (JMA26), *Pspank(hy)-(rapHphrH)* (JMA27), *Pspank(hy)-rapJ* (JMA29), and *Pspank(hy)-rapK* (JMA30) cells were grown in defined minimal medium for at least four generations to an optical density at 600 nm (OD₆₀₀) of ~0.5. IPTG was added to half of the cultures, and samples were collected from induced and uninduced cultures 30 min later. Wild-type (JH642), Δ *phrC* (RSM121), Δ *phrF* (JMA163), and Δ *phrK* (CAL7) cells were grown in defined minimal medium for at least four generations to an OD₆₀₀ of ~1, when samples were collected.

Cells were harvested and total RNA was prepared as described previously (7). RNA from each sample was reverse transcribed and labeled as described previously (2). In the experiments monitoring gene expression in cells overexpressing the indicated *rap* gene, labeled cDNAs from induced samples (with IPTG) and uninduced samples (without IPTG) were cohybridized to cDNA microarrays as described previously (2). In the experiments monitoring gene expression in wild-type, Δ *phrC*, Δ *phrF*, and Δ *phrK* cells, labeled cDNA from each experimental sample was hybridized with a labeled reference cDNA sample to 65-mer oligonucleotide arrays as described previously (2).

Arrays were scanned and analyzed as described previously (2). Iterative outlier analysis (7, 37) was used as described previously (2) to identify genes whose expression changed significantly with 95% or greater confidence. The mean ratio for a set of three independent experiments is reported. Lists of significant genes were arranged into known or putative operons based on the prediction of co-orientation of transcription and the absence of predicted Rho-independent terminators. If a gene or genes that were part of a known or predicted operon changed significantly, the average fold changes in gene expression for the other genes in the operon were also assessed. If the expression of those genes changed similarly but these changes were below the significance threshold of the analysis, the values of these fold changes were included in Fig. 1 and in Table S1 in the supplemental material.

β -Galactosidase assays. The β -galactosidase specific activities of the indicated fusions were assayed as described previously (26). Specific activity was calculated relative to the OD₆₀₀ of the sample. β -Galactosidase specific activity is plotted relative to the OD₆₀₀ of each sample. In each graph, the results from a single experiment are presented and are representative of the results observed in at least two independent experiments.

Microarray data accession number. Complete microarray results are available in Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>). The accession number for the series record is GSE4670.

RESULTS

Characterization of the effects of Rap overproduction on global gene expression. We used DNA microarrays as an initial approach to characterize genes whose mRNA levels were affected by several Rap proteins. We examined the effects of

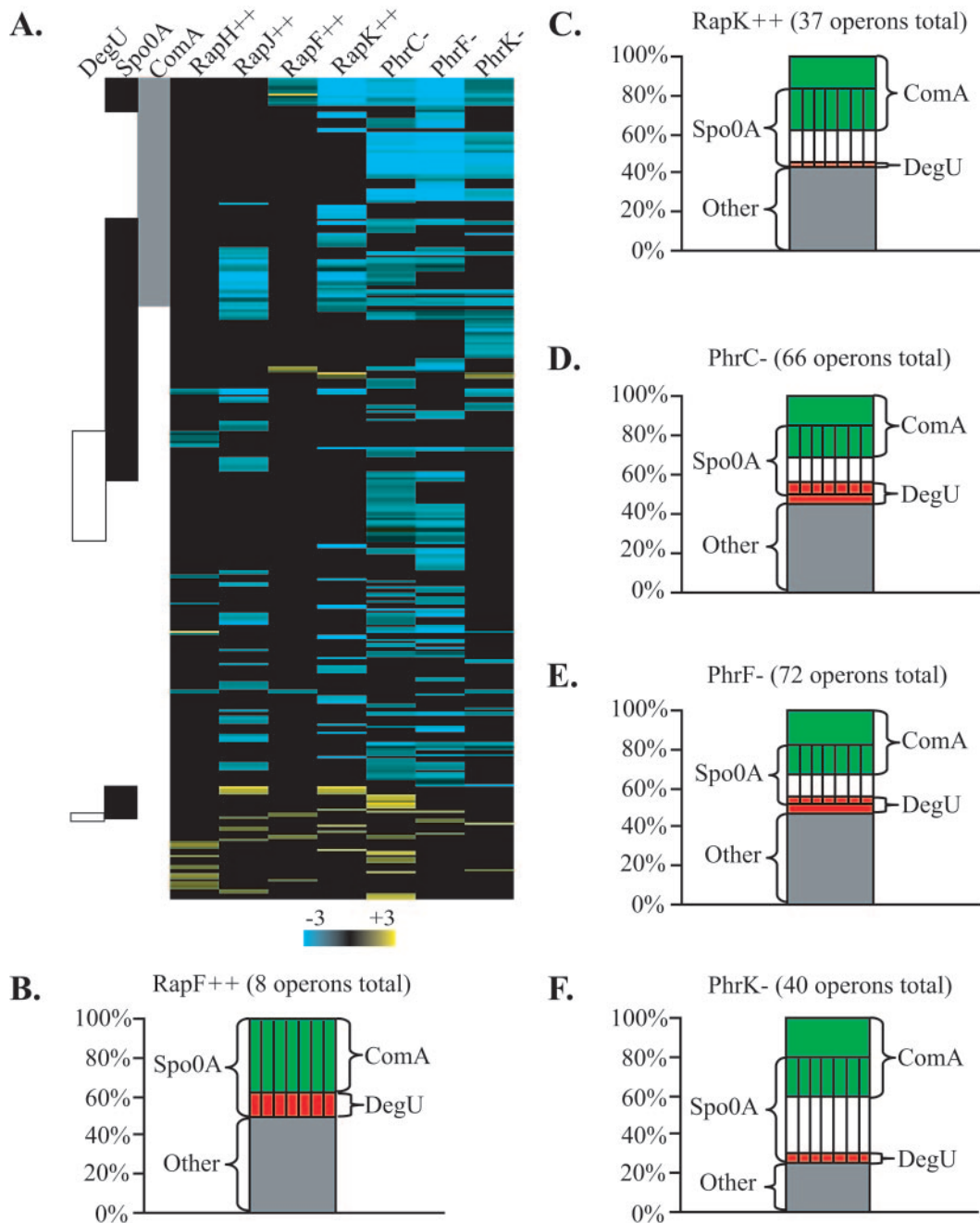


FIG. 1. Overexpression of *rapF* or *rapK* and deletion of *phrC*, *phrF*, or *phrK* inhibit expression of genes activated by ComA. We used DNA microarrays to examine changes in mRNA levels in response to overexpression of *rapF*, *rapH*, *rapJ*, or *rapK* or deletion of *phrC*, *phrF*, or *phrK*. A. Genes whose expression changed significantly in response to overexpression of *rapF* (RapF⁺⁺), *rapJ* (RapJ⁺⁺), or *rapK* (RapK⁺⁺), to cooverexpression of *rapH* and *phrH* (RapH⁺⁺), or to deletion of *phrC* (PhrC⁻), *phrF* (PhrF⁻), or *phrK* (PhrK⁻) were identified as described in Materials and Methods and are represented by a box shaded to represent the magnitude of the mean fold change in gene expression. A threefold or greater decrease in gene expression is shaded bright blue, and a threefold or greater increase in gene expression is shaded bright yellow. Those genes whose expression did not change significantly are shaded black. Additional microarray results, including the gene names and numerical values of the fold changes in gene expression, are in Table S1 in the supplemental material. The boxes to the left of the visualization indicate those genes whose expression were previously shown to be regulated by the response regulators ComA (5, 9, 53, 54) (gray box), Spo0A (14, 46) (black boxes), and DegU (40, 54) (white boxes). B to F. Percentage of operons whose expression changed significantly in response to overexpression of *rapF* or *rapK* or to deletion of *phrC*, *phrF*, or *phrK* and are known to be regulated by the response regulators ComA (green segments), Spo0A (striped segments), DegU (red segments), or other regulators (gray segments). B. Gene expression changes in cells overexpressing *rapF* (RapF⁺⁺). C. Gene expression changes in cells overexpressing *rapK* (RapK⁺⁺). D. Gene expression changes in $\Delta phrC$ cells (PhrC⁻). E. Gene expression changes in $\Delta phrF$ cells (PhrF⁻). F. Gene expression changes in $\Delta phrK$ cells (PhrK⁻).

TABLE 3. Effects of RapF or RapK overproduction or *phrC*, *phrF*, or *phrK* deletion on gene expression

<i>rap</i> or <i>phr</i> modulation	No. of operons:					Other ^a
	Activated by ComA	Activated by Spo0A	Activated by DegU	Repressed by Spo0A	Repressed by DegU	
<i>rapF</i> overexpression	3 ^b	3 ^b		2 ^c	1 ^c	3
<i>rapK</i> overexpression	14 ^d	11 ^{d,e}	1 ^e	4		16
Δ <i>phrC</i>	21 ^f	21 ^{f,g}	7 ^g	2		30
Δ <i>phrF</i>	24 ^h	21 ^{h,i}	5 ⁱ	1	1	34
Δ <i>phrK</i>	16 ^j	22 ^{j,k}	2 ^k			10

^a Operons are regulated by other response regulators or are not known to be regulated by a response regulator.

^b Three operons are activated by both ComA and Spo0A.

^c One operon is repressed by both Spo0A and DegU.

^d Eight operons are activated by both ComA and Spo0A.

^e One operon is activated by both Spo0A and DegU.

^f Eleven operons are activated by both ComA and Spo0A.

^g Four operons are activated by both Spo0A and DegU.

^h Eleven operons are activated by both ComA and Spo0A.

ⁱ Three operons are activated by both Spo0A and DegU.

^j Eight operons are activated by both ComA and Spo0A.

^k Two operons are activated by both Spo0A and DegU.

rapF, *rapH*, *rapJ*, and *rapK* overexpression on global mRNA levels. Although changes in global mRNA levels may result from changes in the level of gene transcription as well as changes in the levels of RNA stability, for simplicity we assumed that changes in mRNA levels reflect changes in gene expression. This same microarray-based strategy successfully elucidated the role that RapI plays in activating expression of genes in the ICEBsI mobile element (2).

Each *rap* gene was overexpressed from the IPTG-inducible promoter *Pspank(hy)* during exponential growth in defined minimal medium. RNA transcript levels in induced and uninduced cells were compared 30 min after induction. We analyzed the results of three independent experiments to identify those genes whose expression changed significantly in response to overproduction of each Rap. We compared the results of these experiments to the published genomewide analyses of several response regulator regulons (9, 14, 29, 40, 46, 54) in order to identify response regulators whose activities were potentially regulated by these Rap proteins. In particular, we were interested in identifying any Rap proteins that affect ComA-dependent gene expression.

Overproduction of each Rap protein resulted in changes in the expression of several genes. RapH overproduction caused small changes in expression of 15 genes; there was no significant overlap between those genes affected by RapH overproduction and characterized response regulator regulons (Fig. 1A; see Table S1 in the supplemental material). These observations are in contrast to the results recently reported by Hayashi et al., who observed that *rapH* overexpression inhibited *srfA*, *rapF*, and *sacB* expression (24). Reanalysis of the nucleotide sequence of our *rapH* overexpression construct, which was constructed based on the previously published sequence for the *rapH* region (30) and not the newly reported sequence of Hayashi et al. which identified multiple sequence errors in this region (24), revealed that our construct overexpresses both *rapH* and *phrH*. Therefore, we believe that the small changes in gene expression that were observed were due to efficient inhibition of RapH's activity by the coexpressed PhrH peptide.

RapJ overproduction affected the expression of 39 operons, including 20 that are known to be regulated by Spo0A. Expres-

sion of 17 Spo0A-activated operons decreased in cells overproducing RapJ, and expression of 3 Spo0A-repressed operons increased (Fig. 1A; see Table S1 in the supplemental material). It is not known whether RapJ directly or indirectly inhibits the activity of Spo0A; further studies will be needed to determine how RapJ affects Spo0A-dependent gene expression.

Overproduction of RapF or RapK inhibited the expression of genes known to be activated by ComA (Fig. 1A to C and Table 3). The effects of RapF overproduction on ComA-dependent gene expression are consistent with previously published data (6). To understand how multiple Rap proteins and Phr peptides affect the activity of ComA, we further explored the roles that RapF, RapK, PhrF, and PhrK play in regulating ComA-dependent gene expression.

Effects of RapF overproduction on gene expression. Overproduction of RapF had few effects on gene expression, with significant changes in only eight operons (Fig. 1A and B and Table 3; see Table S1 in the supplemental material). Three of the eight operons affected are directly activated by ComA, whereas the remaining operons are regulated by other response regulators or their regulation is unknown. Although *rapF* overexpression inhibited only a small subset of the ComA regulon, we conclude that overexpression of RapF inhibits ComA-dependent gene expression, because the genes that were inhibited show some of the largest changes in gene expression in response to perturbations in the levels of *comA* (9). The modest effects of RapF overproduction on gene expression may be partially explained by the effects of the negative autoregulatory loop that controls expression of *rapF* from its native copy in the chromosome. Transcription from the *rapF* promoter is activated by ComA (9) and should be partially repressed in response to increased RapF activity, resulting in a smaller net increase in the amount of active RapF upon *rapF* overexpression from the inducible promoter. Consistent with this, we observed that *rapF* transcripts increased less than threefold in response to induction from the IPTG-dependent promoter. In addition, modest effects of *rapF* overexpression on ComA-dependent genes may be partially explained by the time at which gene expression was assayed (OD₆₀₀ of ~0.8), when expression of ComA-dependent genes is still increasing

to its maximal level. Gene expression changes were measured under these conditions to avoid analyzing cells transitioning from exponential-phase growth to stationary-phase growth, when patterns of gene expression change dramatically. RapF may also affect transcription independently of ComA, because four of the operons whose expression changed in response to RapF overproduction are not regulated by ComA.

Effects of RapK overproduction on gene expression. The expression of 37 operons changed significantly in response to RapK overproduction (Fig. 1A and C and Table 3; see Table S1 in the supplemental material). Fourteen of the 30 operons whose expression decreased are activated by ComA, including eight operons that are also activated by Spo0A. RapK overproduction inhibited the expression of three additional operons that are activated by Spo0A and activated expression of four operons that are repressed by Spo0A. Based on these results, we infer that RapK inhibits the activity of ComA, either directly or indirectly, as the majority of operons whose expression demonstrated the largest decreases in expression are activated directly by ComA and indirectly by Spo0A (see Table S1 in the supplemental material). However, several genes whose expression changed in response to overexpression of *rapK* are not part of the ComA regulon (Fig. 1A and C and Table 3; see Table S1 in the supplemental material), indicating that RapK likely affects the activity of additional regulators. Spo0A is likely another direct or indirect target of RapK, as several of the operons affected by *rapK* overexpression are known to be regulated by Spo0A. Nevertheless, the effect of RapK overproduction on the expression of ComA-regulated genes is unlikely to be due solely to effects on Spo0A's activity, as many of the ComA-regulated genes whose expression changed in response to overproduction of RapK did not change in response to overproduction of RapJ, which also inhibited Spo0A-dependent gene expression (Fig. 1A; see Table S1 in the supplemental material).

Overproduction of RapF and RapK inhibits expression of *Ppel-lacZ*. In order to further investigate the roles that RapF and RapK play in regulating ComA-dependent gene expression, we monitored the effects of RapF and RapK overproduction on the expression of the ComA-activated gene *pel* by using a fusion of the *pel* promoter to the reporter gene, *lacZ*. In contrast to the microarray experiments, which monitor gene expression at only a single time, the *Ppel-lacZ* fusion provides a dynamic picture of the effects of RapF or RapK overproduction on gene expression throughout growth. We compared expression of *Ppel-lacZ* in cells overexpressing *rapF* or *rapK* to that in control cells with an empty overexpression vector (Fig. 2). Consistent with previous observations (9), *pel* expression was initially low and increased with increasing cell density in control cells (Fig. 2). Other ComA-regulated genes exhibit similar patterns of expression (32, 41) (Fig. 3). Overproduction of RapF or RapK prevented the density-dependent increase in *pel* expression (Fig. 2). A similar effect was observed when *rapC*, the other known ComA inhibitor, was overexpressed (Fig. 2). These data further demonstrate that RapF and RapK inhibit ComA-dependent gene expression when overproduced and indicate that RapF and RapK could play significant roles in regulating ComA-dependent gene expression.

PhrC, PhrF, and PhrK stimulate ComA-dependent gene expression. All characterized Rap proteins are inhibited by

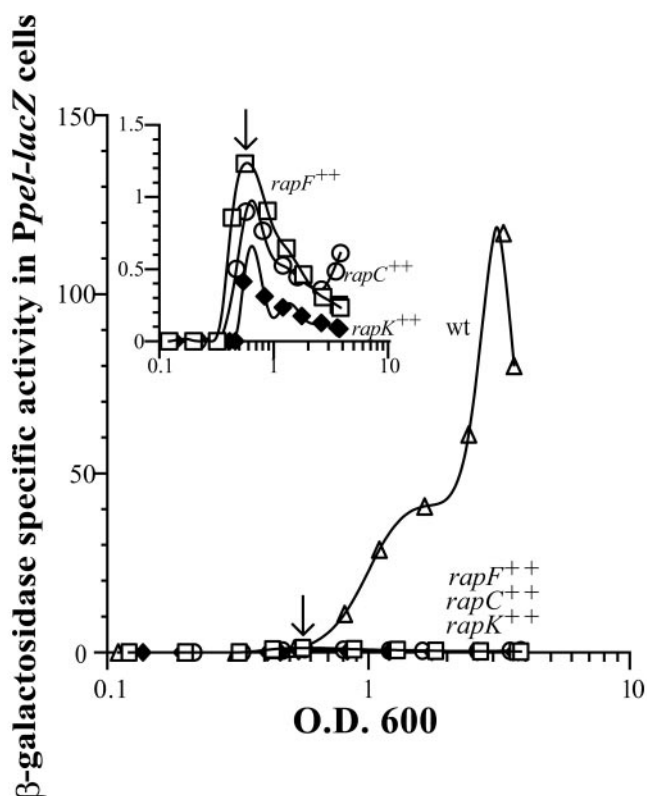


FIG. 2. Overexpression of *rapC*, *rapF*, or *rapK* inhibits expression of the ComA-activated gene *pel*. Cells containing *Ppel-lacZ* and *Pspank(hy)-rapC* (JMA78), *Pspank(hy)-rapF* (JMA76), *Pspank(hy)-rapK* (JMA77), or *Pspank(hy)* (JMA79) were grown in defined minimal medium. IPTG was added to cells at an OD_{600} of ~ 0.4 to 0.6 . Samples were collected from cells prior to IPTG addition, at the time of IPTG addition, and 30, 60, 90, 120, 150, and 180 min. after IPTG addition. β -Galactosidase activity was assayed as described in Materials and Methods and is plotted relative to the OD_{600} values of the samples. The arrow indicates the time of IPTG addition. *Ppel-lacZ* expression in *Pspank(hy)* (Δ) (wild type [wt]), *Pspank(hy)-rapC* (\circ) (*rapC*⁺⁺⁺), *Pspank(hy)-rapF* (\square) (*rapF*⁺⁺⁺), and *Pspank(hy)-rapK* (\blacklozenge) (*rapK*⁺⁺⁺) cells is shown. The inset shows data replotted with the y axis from 0 to 1.5 units of β -galactosidase specific activity.

their cognate Phr peptides. Therefore, we examined the roles that PhrF and PhrK play in regulating the expression of ComA-regulated genes. We used DNA microarrays to compare mRNA levels in $\Delta phrF$ and $\Delta phrK$ mutants to mRNA levels in wild-type cells. We expected that loss of *phrF* or *phrK* would result in decreased ComA-dependent gene expression, as the activity of RapF or RapK should increase due to the absence of their inhibitory peptides. We also tested the effects of deletion of *phrC* on global gene expression. The expression of several ComA-regulated genes (*srfA*, *rapA*, and *rapC*) is known to decrease in $\Delta phrC$ mutants (10, 32, 70); this is due to increased RapC activity (10, 70). We found that deletion of all three *phr* genes resulted in decreased expression of genes activated by ComA, with $\Delta phrF$ and $\Delta phrK$ mutants having the largest and smallest decreases in magnitude of expression of ComA-dependent genes, respectively (Fig. 1A and D to F; see Table S1 in the supplemental material).

(i) $\Delta phrC$. Deletion of *phrC* significantly changed the expression of 66 operons (Fig. 1A and D and Table 3; see Table S1

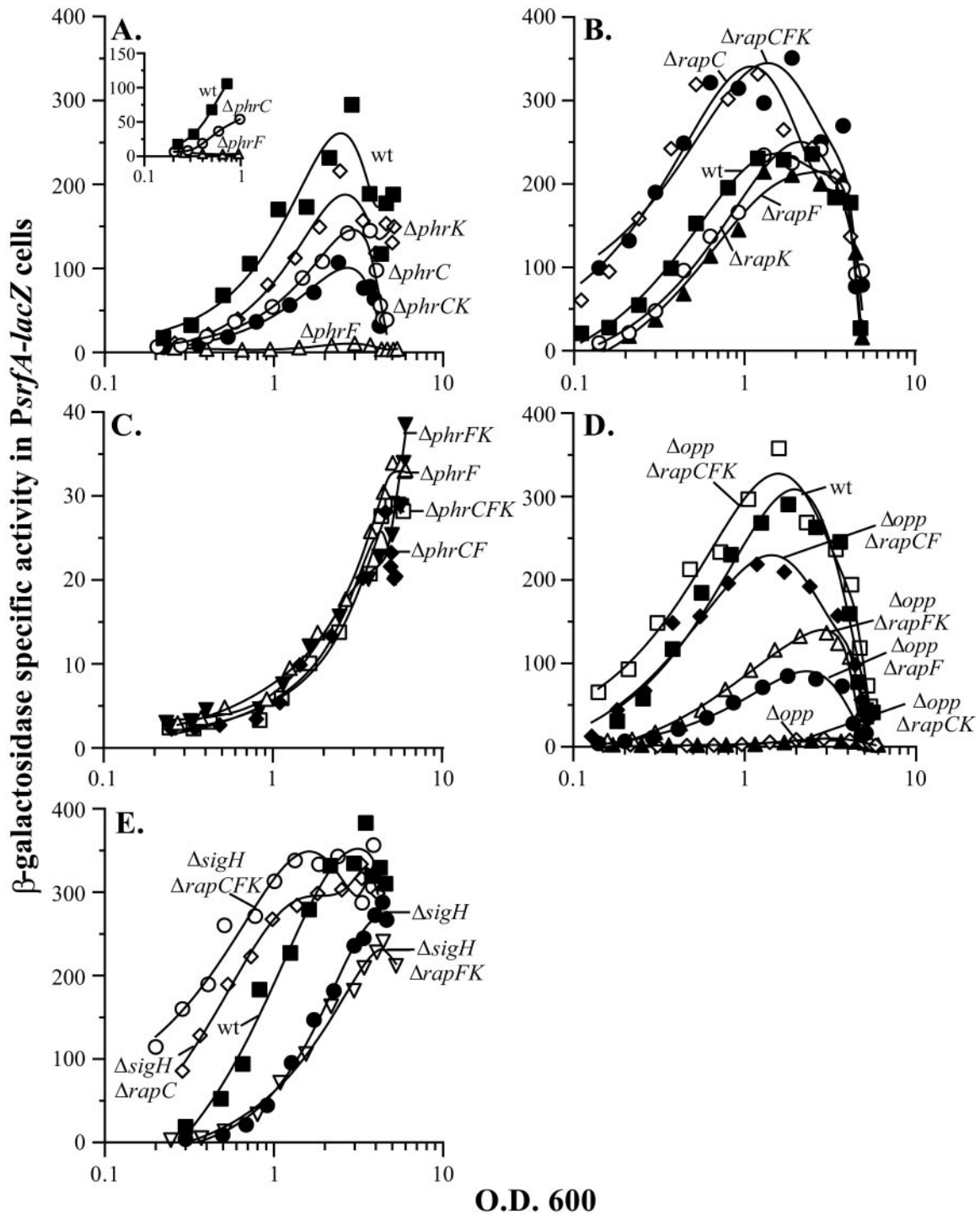


FIG. 3. Effects of *rap* and *phr* deletions on expression of *srfA*. *PstfA-lacZ*-containing cells were grown in defined minimal medium, and samples were removed throughout growth for determination of β -galactosidase specific activity. β -Galactosidase specific activity was determined as described in Materials and Methods and is plotted relative to the OD_{600} values of the samples. A. Wild-type (wt) (JMS682) (■), $\Delta phrK$ (CAL8) (\diamond), $\Delta phrC$ (JMA165) (\circ), $\Delta phrC \Delta phrK$ ($\Delta phrCK$) (CAL9) (\bullet), and $\Delta phrF$ (JMA 166) (\triangle) cells. The inset shows data from the early time points (OD_{600} of <1) in wild-type, $\Delta phrC$, and $\Delta phrF$ cells replotted with a y axis from 0 to 150 units of β -galactosidase specific activity. B. Wild-type (JMS682) (■), $\Delta rapC$ (JMA47) (\diamond), $\Delta(rapF phrF)$ ($\Delta rapF$) (JMA117) (\blacktriangle), $\Delta rapK$ (JMA48) (\circ), and $\Delta rapC \Delta(rapF phrF) \Delta rapK$ ($\Delta rapCFK$) (JMA142) (\bullet) cells. C. $\Delta phrF$ (\triangle) (JMA166), $\Delta phrC \Delta phrF$ ($\Delta phrCF$) (JMA169) (\blacklozenge), $\Delta phrF \Delta phrK$ ($\Delta phrFK$) (CAL10) (\blacktriangledown), and $\Delta phrC \Delta phrF \Delta phrK$ ($\Delta phrCFK$) (CAL11) (\square) cells. D. Wild-type (JMA682) (■), Δopp (JMA52) (\diamond), $\Delta opp \Delta(rapF phrF)$ ($\Delta opp \Delta rapF$) (JMA122) (\bullet), $\Delta opp \Delta rapC \Delta(rapF phrF)$ ($\Delta opp \Delta rapCF$) (JMA134) (\blacklozenge), $\Delta opp \Delta rapC \Delta rapK$ ($\Delta opp \Delta rapCK$) (JMA58) (\blacktriangle), $\Delta opp \Delta(rapF phrF) \Delta rapK$ ($\Delta opp \Delta rapFK$) (JMA138) (\triangle), and $\Delta opp \Delta rapC \Delta(rapF phrF) \Delta rapK$ ($\Delta opp \Delta rapCFK$) (JMA144) (\square) cells. E. Wild-type (JMS682) (■), $\Delta sigH$ (JMA51) (\bullet), $\Delta sigH \Delta rapC$ (JMA53) (\diamond), $\Delta sigH \Delta(rapF phrF) \Delta rapK$ ($\Delta sigH \Delta rapFK$) (JMA139) (\blacktriangledown), and $\Delta sigH \Delta rapC \Delta(rapF phrF) \Delta rapK$ ($\Delta sigH \Delta rapCFK$) (JMA149) (\circ) cells.

in the supplemental material). Twenty-one of the 54 operons whose expression decreased are activated by ComA; these include 11 operons that are activated by Spo0A. Deletion of *phrC* also resulted in decreased expression of 10 additional operons that are activated by Spo0A. The remaining operons whose expression changed in response to deletion of *phrC* were either regulated by other response regulators or not known to be regulated by a response regulator (Fig. 1A and D and Table 3; see Table S1 in the supplemental material).

(ii) ***ΔphrF***. Deletion of *phrF* resulted in significant changes in the expression of 72 operons (Fig. 1A and E and Table 3; see Table S1 in the supplemental material). Twenty-four of the 69 operons whose expression decreased in *ΔphrF* cells are known to be activated by ComA; these include 11 that are also activated by Spo0A. Deletion of *phrF* also resulted in decreased expression of 10 additional operons that are activated by Spo0A. The remaining operons whose expression changed in response to deletion of *phrF* were either regulated by other response regulators or not known to be regulated by a response regulator (Fig. 1A and E and Table 3; see Table S1 in the supplemental material).

(iii) ***ΔphrK***. Deletion of *phrK* resulted in significant changes in the expression of 40 operons (Fig. 1A and F and Table 3; see Table S1 in the supplemental material). Of the 38 operons whose expression decreased in *ΔphrK* cells, 16 are activated by ComA, including 8 that are also activated by Spo0A. Deletion of *phrK* resulted in decreased expression of 14 additional operons that are activated by Spo0A, whereas the remaining operons affected by deletion of *phrK* are not known to be regulated by response regulators (Fig. 1A and F and Table 3; see Table S1 in the supplemental material).

Targets for Phr peptides and Rap proteins. Consistent with previous studies and our results from overproduction of RapF and RapK, our analysis of *ΔphrC*, *ΔphrF*, and *ΔphrK* mutants demonstrates that all three Phr peptides play a role in activating expression of genes regulated by ComA. PhrC and PhrF are known to stimulate the expression of ComA-dependent genes by inhibiting RapC and RapF, respectively (6, 70). RapC and RapF have been previously shown to directly interact with ComA and to inhibit its ability to bind to DNA (6, 10). PhrK likely stimulates ComA-dependent gene expression by inhibiting the activity of RapK. RapK inhibits ComA-dependent gene expression either by acting on ComA directly or by affecting the activity of another regulator that is known to modulate ComA-dependent gene expression (14, 20, 32, 46, 67).

RapK may also play a role in directly or indirectly regulating the expression of genes activated by Spo0A, because a number of Spo0A-controlled genes were affected by overproduction of RapK and deletion of *phrK*. There are two classes of Spo0A-regulated genes: those genes whose transcription is activated or repressed by intermediate levels of phosphorylated Spo0A (Spo0A~P), which include genes involved in biofilm formation, competence development, and antibiotic production, and those genes whose transcription is activated or repressed by high levels of Spo0A~P, many of which are involved in sporulation (15, 16, 20–22). This regulation is thought to allow cells to progressively respond to decreasing nutrient availability as the amount of Spo0A~P increases (8, 15, 16). Under the conditions tested, we expected that the majority of genes whose expression would change would be those that were reg-

ulated by intermediate levels of Spo0A~P. This is what we observed, as the majority of Spo0A-regulated genes whose expression changed in response to overexpression of *rapK* or deletion of *phrK* were those that respond to intermediate levels of Spo0A~P. Further work is needed to determine if RapK can inhibit Spo0A under conditions favorable for regulation of genes dependent upon high levels of Spo0A~P.

Although one might expect that overexpression of each *rap* gene and deletion of each cognate *phr* would have similar effects on gene expression, this is not what we observed. This is likely due to the levels of active Rap protein that were present in each condition. Overexpression of *rapF* had very modest effects on global gene expression, whereas deletion of *phrF* had large effects on gene expression. As described above, the modest effects of RapF overproduction on global gene expression were likely due to decreased expression from the native copy of *rapF*, resulting in a relatively small increase in the ratio of RapF to PhrF. In contrast, any RapF protein that was produced in *phrF* mutant cells should be active, as it is in excess over its inhibitory peptide. Although overexpression of *rapK* had a larger effect on ComA-dependent gene expression than did deletion of *phrK*, these results may be partially explained by the fact that transcription of *rapK* from its native promoter was likely low at the time at which changes in gene expression were measured, as expression of *rapK* is thought to be indirectly activated by Spo0A, which activates expression of genes at the end of exponential phase and in early stationary phase (14, 17). This regulation likely explains most of the differences in the magnitude of effects observed in response to overexpression of *rapF* or *rapK* and deletion of *phrF* or *phrK*.

In addition to genes regulated by ComA, deletion of each *phr* gene also resulted in changes in the expression of several other genes. Many of these genes were not detected in the *rap* overexpression experiments. These differentially regulated genes may be regulated by both the *rap* and *phr* genes and reflect differences in the way the experiments were performed, i.e., transient overexpression of each *rap* gene compared to absence of each *phr* gene throughout growth. Alternatively, each Phr peptide may affect the activity of a protein or proteins in addition to its cognate Rap protein. The PhrC and PhrG peptides affect the activities of proteins in addition to their cognate Rap proteins (52, 55, 70). PhrC interacts with two additional proteins (70), one of which is the indirect Spo0A antagonist RapB (55). Therefore, the effects of the *ΔphrC* mutation on Spo0A-regulated gene expression may reflect uninhibited activity of RapB. The other target of PhrC has not been identified, so it is possible that some of the gene expression changes observed in the *ΔphrC* mutant could reflect changes in the activity of this protein.

PhrC, PhrF, and PhrK play different roles in stimulating ComA-dependent gene expression. The results of our microarray analysis of the *ΔphrC*, *ΔphrF*, and *ΔphrK* mutants indicated that the *ΔphrF* mutation had the largest effect on the expression of genes activated by ComA under the conditions tested, i.e., mid-to-late exponential growth in defined minimal medium (OD₆₀₀ of ~1). However, as peptide levels change throughout growth, possibly affecting gene expression to different extents, we used a fusion of *lacZ* to the ComA-dependent promoter *srfA* (*PsrfA-lacZ*) to monitor the effects of *phr* null mutations throughout growth (Fig. 3A).

As described previously (70), we found that *srfA* expression was ~2-fold lower in $\Delta phrC$ mutants than in the wild type and that the initial increase in *srfA* expression was slightly delayed (Fig. 3A). In $\Delta phrF$ mutant cells, *srfA* expression was reduced ~10- to 20-fold and the initial increase in *srfA* expression was delayed several generations relative to that in wild-type cells. Furthermore, significant differences in the level of *srfA* expression were observed in $\Delta phrF$ cells at lower cell densities, a condition where there were much smaller differences in the magnitude of *srfA* expression between wild-type, $\Delta phrC$, and $\Delta phrF$ cells (Fig. 3A, inset). In the $\Delta phrK$ mutants, *srfA* expression was ~75% of that in wild-type cells and was slightly delayed.

These results provide further evidence that all three Phr peptides are required for full levels of ComA-dependent gene expression and that PhrC, PhrF, and PhrK stimulate ComA-dependent gene expression to different magnitudes. Furthermore, the effects of the *phrC*, *phrF*, and *phrK* mutations on *srfA* expression were dependent upon the presence of their cognate *rap* genes, as *srfA* expression was not inhibited when both *rap* and *phr* were inactivated [$\Delta(rapF\ phrF)$ (Fig. 3B) and $\Delta rapK\ \Delta phrK$ (data not shown)]. These observations, as well as previously published results (70), indicate that ComA-dependent gene expression decreases in $\Delta phrC$, $\Delta phrF$, and $\Delta phrK$ mutants due to the increased activities of RapC, RapF, and RapK, providing further evidence that RapC, RapF, and RapK regulate ComA-dependent gene expression.

Multiple Phr peptides act independently to inhibit *srfA* expression. We also examined the effects of multiple *phr* deletions on *srfA* expression to determine if each *phr* gene stimulated ComA-dependent gene expression independently. We found that *srfA* expression was less in $\Delta phrC\ \Delta phrK$ double mutant cells than in $\Delta phrC$ or $\Delta phrK$ single mutants (Fig. 3A), indicating that PhrC and PhrK act independently to stimulate ComA-dependent gene expression. Adding additional *phr* mutations ($\Delta phrC$, $\Delta phrK$, or $\Delta phrC$ and $\Delta phrK$) to the $\Delta phrF$ cells had little effect on *srfA* expression (Fig. 3C), indicating that in the absence of *phrF*, neither PhrC nor PhrK significantly stimulates ComA-dependent gene expression. These data provide further evidence of the important role that PhrF plays in stimulating ComA-dependent gene expression and the modest roles played by the PhrC and PhrK peptides.

Deletion of *rapF* or *rapK* has no effect on ComA-dependent gene expression. As overexpression of *rapF* or *rapK* or deletion of *phrF* or *phrK* resulted in decreased ComA-dependent gene expression, we monitored the effects of deletion of *rapF* or *rapK* on ComA-dependent gene expression. Deletion of *rapC* in otherwise wild-type cells results in increased expression of ComA-dependent genes (10, 70). However, we found that deletion of *rapF* or *rapK* had no detectable effect on *srfA* expression under the conditions tested: growth in minimal medium (Fig. 3B), nutrient broth sporulation medium (DSM) (data not shown), or DSM supplemented with glucose and glutamate (described previously [11]; data not shown). Similarly, Bongiorno et al. (6) did not observe changes in expression of the ComA-dependent gene *rapA* in $\Delta rapF$ mutant cells.

We were also unable to detect additional effects on *srfA* expression when the $\Delta rapC$ mutation was combined with the $\Delta rapF$, $\Delta rapK$, or both the $\Delta rapF$ and $\Delta rapK$ mutations (Fig. 3B and data not shown). This is in contrast to the results reported

by Bongiorno et al. (6), who observed that combining the $\Delta rapF$ and $\Delta rapC$ mutations resulted in a further increase in expression of *rapA*. These conflicting observations could be due to differences in the promoters assayed (*rapA* compared to *srfA*) or the growth conditions (DSM, in which ComA-dependent gene expression increases at the end of exponential growth, versus minimal glucose medium, in which ComA-dependent gene expression increases during exponential growth) (41, 51). However, because we observed that overproduction of RapF or RapK or deletion of PhrF or PhrK results in decreased ComA-dependent gene expression, we suspect that RapF and RapK have roles in regulating ComA-dependent gene expression under conditions that we have not yet identified.

Rap proteins inhibit ComA-dependent gene expression in strains defective for synthesis and uptake of Phr peptides. The inability to detect effects of $\Delta rapF$ and $\Delta rapK$ mutations on *srfA* expression in cells grown in minimal media was surprising, as our previous results indicated that under these growth conditions, RapC, RapF, and RapK all actively repress *srfA* expression in the absence of their inhibitory peptides. Therefore, we looked for additional insights into the roles that RapC, RapF, and RapK play in regulating ComA-dependent gene expression by examining the effects of *rapC*, *rapF*, and *rapK* mutations in the presence of mutations, Δopp or $\Delta sigH$, that affect ComA-dependent gene expression, likely by affecting import and synthesis of Phr peptides, respectively.

(i) **Δopp .** Previous work had shown that the oligopeptide permease (Opp) is required for competence development, sporulation, and expression of *srfA* (20, 65). The requirement for *opp* for sporulation is bypassed by deleting both *rapA* and *rapB*, indicating that the major (and probably only) role for Opp in sporulation is to inhibit the activities of the RapA and RapB proteins, likely by importing the PhrA and PhrC peptides, respectively (59). However, the role for Opp in competence development and *srfA* expression is less clear. Null mutations in *rapC* do not bypass the need for *opp* for expression of *srfA* or competence development. It seemed likely that *opp* null mutants had decreased expression of ComA-activated genes due to uninhibited activity of Rap proteins other than or in addition to RapC (34, 70).

Based on our observations that RapC, RapF, and RapK all inhibit expression of *srfA* and other genes activated by ComA, we thought it was likely that the role that Opp played in regulating *srfA* expression was to import the peptides that antagonize the activities of RapC, RapF, and RapK. Therefore, we tested the ability of *rapC*, *rapF*, and *rapK* deletions to suppress the defects in *srfA* expression that occurred in an *opp* mutant. We found that deletion of *rapC*, *rapK*, or both *rapC* and *rapK* was not able to suppress the defect in *srfA* expression observed in Δopp cells, as *srfA* was expressed at the same low level in Δopp , $\Delta opp\ \Delta rapC$, $\Delta opp\ \Delta rapK$, and $\Delta opp\ \Delta rapC\ \Delta rapK$ mutant cells (Fig. 3D and data not shown). However, deletion of *rapF* in Δopp mutant cells resulted in a significant restoration of *srfA* expression (Fig. 3D). *srfA* expression was further enhanced in $\Delta opp\ \Delta rapF$ cells by deletion of *rapC*, *rapK*, or both *rapC* and *rapK* (Fig. 3D). *srfA* expression in $\Delta opp\ \Delta rapC\ \Delta rapF\ \Delta rapK$ cells was slightly higher than that in wild-type cells.

These results indicate that the primary reason that *opp* mutant cells exhibit low levels of *srfA* expression is due to unin-

hibited activity of RapF. In addition, the similarly low levels of *srfA* expression in $\Delta phrF$ and Δopp mutant cells indicate that the increase in RapF activity in *opp* mutant cells is primarily due to the inability to import the PhrF peptide. The changes in the timing and level of *srfA* expression in the $\Delta opp \Delta rapF \Delta rapK$ and $\Delta opp \Delta rapC \Delta rapF$ mutants also correlate well with the changes in the timing and level of *srfA* expression in the $\Delta phrC$ and $\Delta phrK$ mutants. Taken together, these results indicate that in the absence of Opp and the import of the PhrC, PhrF, and PhrK peptides, RapF, and to a lesser extent RapC and RapK, inhibit ComA-dependent gene expression.

(ii) **$\Delta sigH$.** σ^H directs some of the transcription of all *phr* genes except *phrA* and *phrH* (24, 32, 42, 47). σ^H is also required for full activation of *srfA* expression (26) and seems to play a role in the posttranslational production of mature PhrC peptide (32). We tested whether the decrease in *srfA* expression that occurs in *sigH* mutants was due to the increased activity of RapC, RapF, or RapK as a result of decreased inhibition by their cognate peptides. We found that deletion of *rapF*, *rapK*, or both *rapF* and *rapK* in $\Delta sigH$ mutants had no effect on *srfA* expression (Fig. 3E and data not shown). However, deletion of *rapC* in $\Delta sigH$ mutants restored *srfA* expression to levels slightly higher than wild-type levels (Fig. 3E). Deletion of *rapF* or *rapK* in addition to *rapC* in $\Delta sigH$ cells resulted in levels of expression similar to those as observed in $\Delta sigH \Delta rapC$ cells (data not shown), whereas deletion of *rapC*, *rapF*, and *rapK* in $\Delta sigH$ mutants resulted in a further increase in the levels of *srfA* expression (Fig. 3E). These results indicate that the primary defect in *srfA* expression in *sigH* mutants is due to increased RapC activity but that increased RapF and RapK activities also contribute to the lower levels of *srfA* expression in *sigH* mutant cells in the absence of RapC.

These results, compared to the results observed with deletion of *rap* genes in *opp* mutant cells, also indicate that there may be different requirements for σ^H activity among the Phr peptides, with some Phr peptides being more dependent upon σ^H activity, at the level of either *phr* transcription or posttranslational processing. *phrA* and *phrH* do not possess σ^H -dependent promoters and instead appear to be transcribed only from the upstream *rap* promoters (24, 42, 59). *phrE* and *phrI* are transcribed from at least one promoter in addition to the σ^H -dependent promoter (42). As the role that σ^H plays in the posttranslational processing of PhrC and potentially other peptides has not been elucidated, it is currently not clear whether different Phr peptides may be more dependent upon σ^H activity for posttranslational processing.

DISCUSSION

PhrK stimulates ComA-dependent gene expression by antagonizing RapK. In this work, we identified an additional *rap-phr* pair, *rapK-phrK*, that regulates the expression of several genes activated by the response regulator ComA (9). We observed that PhrK stimulates expression of genes activated by ComA by antagonizing the activity of RapK. Since other regulatory proteins are also known to regulate transcription of some of the genes that are activated by ComA (14, 20, 33, 46, 67), the effects mediated by RapK and PhrK may occur through direct inhibition of ComA activity by RapK or through inhibition of the activity of another regulatory protein. *rapK*

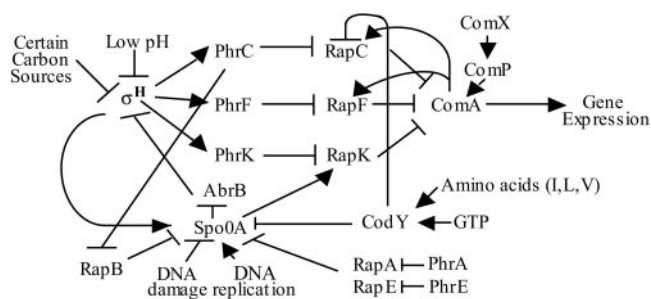


FIG. 4. Integration of multiple signals for complex regulation of ComA-dependent gene expression. This diagram illustrates several of the physiological cues that are known to influence ComA-dependent gene expression. Further details are provided in the text.

transcription is thought to be activated indirectly by Spo0A (14, 46). Therefore, regulation of ComA-dependent gene expression by RapK provides an opportunity for additional signals, such as those that regulate Spo0A activity, to be incorporated into the decision to activate ComA-dependent gene expression (Fig. 4).

RapC, RapF, and RapK play different roles in regulating ComA activity. We also observed that RapC, RapF, and RapK have different roles in the regulation of ComA-dependent gene expression. We found that RapK has a modest role in inhibiting the expression of genes in the ComA regulon and that under the conditions tested, this is evident only when the levels of RapK exceed the levels of PhrK. RapF is a potent antagonist of ComA-dependent gene expression in the absence of its inhibitory peptide, but there is little evidence for its role in regulating ComA-dependent gene expression when PhrF is produced and able to enter the cell. This is in contrast to the case for RapC, whose modest inhibition of ComA-dependent gene expression is apparent in the presence and absence of PhrC.

The reason that RapF has such profound effects on ComA-dependent gene expression in the absence of PhrF, but not in its presence, may be due to higher intracellular levels of the PhrF peptide at low cell densities, higher sensitivity of RapF protein to low levels of PhrF, a combination of these two factors, or an additional factor that inhibits the activity of RapF under these conditions. If PhrF peptides accumulate in the cell at a lower cell density than PhrC peptides, then RapF activity could be inhibited at lower cell densities than those observed for RapC. Accumulation of PhrF peptides at lower cell density could be due to higher levels of PhrF transcription, processing, or import into the cell. Differences in the transcription or processing of PhrC and PhrF may explain the differences in the sensitivity of *sigH* mutants to the activities of RapC and RapF (Fig. 3E); differences in the import of Phr peptides may explain the observation that mutations in the oligopeptide permease that do not respond to the PhrC peptide but still allow more significant levels of ComA-dependent gene expression than in Δopp cells can be obtained (69). Similarly, if RapF were more sensitive to low levels of Phr peptide than RapC, then RapF's activity could be inhibited at lower cell densities than those observed for RapC. Consistent with these hypotheses, we observed that *srfA* expression was lower in $\Delta phrF$ mutants than in $\Delta phrC$ mutants or wild-type cells at the earliest time points assayed (Fig. 3A, inset).

Integration of multiple signals for complex regulation of gene expression. The involvement of multiple peptides in the activation of ComA provides the opportunity for a variety of physiological signals to modulate the magnitude and timing of ComA-dependent gene expression (Fig. 4). Although ComX production appears to occur at a consistent rate throughout growth (3), the abundance of Rap proteins and Phr peptides is regulated at the level of transcription by proteins that respond to different cellular signals (Fig. 4). Transcription of *rapC* and *rapF* is activated by ComA (9, 32, 54), which establishes a negative autoregulatory loop for transcription (Fig. 4). *rapC* transcription is also repressed by CodY, a protein that is active when cellular pools of branched-chain amino acids and GTP are high (reviewed in reference 72) (Fig. 4). *rapK* is thought to be activated indirectly by Spo0A (14, 46). Several factors are known to regulate the transcription and activity of Spo0A: CodY represses transcription of Spo0A, RNA polymerase containing σ^H provides additional transcription of Spo0A, high population density signals and ongoing DNA replication stimulate the activation of Spo0A, and DNA damage inhibits the activity of Spo0A (reviewed in references 8 and 17) (Fig. 4). Furthermore, transcription from σ^H -dependent promoters affects the levels of *phrC*, *phrF*, and *phrK* transcripts (32, 42, 47), in addition to the factors that regulate expression of their upstream *rap* genes (Fig. 4). σ^H is also regulated at the level of transcription and activity by a variety of physiological signals, including indirect activation of *sigH* transcription by Spo0A and inhibition of σ^H activity by certain carbon sources and low pH (reviewed in reference 8) (Fig. 4).

Differential regulation could also occur during production of the active PhrC, PhrF, and PhrK peptides. Processing of Phr peptides to their mature forms is thought to rely upon the secretion machinery and at least one extracellular protease (31, 56, 75); these proteins may exhibit different specificities towards each peptide. In addition, differential import into the cell through the oligopeptide permease may also contribute to variation in the ability of Phr peptides to antagonize the activities of their partner Rap proteins. Therefore, it is likely that this regulatory network serves to modulate the magnitude and timing of the ComA response under a variety of different conditions.

Similarly, the activity of the Spo0A protein, which activates expression of genes involved in sporulation and other post-exponential-phase processes, is regulated indirectly by multiple Phr peptides that act through Rap proteins that inhibit the activity of Spo0F (27, 55, 57, 59). The involvement of multiple Rap proteins and Phr peptides allows integration of additional signals into this regulatory network, as *rapA* and *rapE* expression is controlled by ComA, *rapB* is expressed during exponential phase, and transcription of *phrC* and *phrE* is stimulated by RNA polymerase containing σ^H (27, 32, 42, 47, 49, 57).

The involvement of multiple quorum-sensing signals in coordinating biological responses is not unique to *B. subtilis*. In *Pseudomonas aeruginosa*, a complex network involving at least three quorum-sensing signals modulates expression of several virulence genes (reviewed in references 28, 68, and 80). In several of the *Vibrio* species, two or three quorum-sensing signals control specific biological responses, including bioluminescence in *V. harveyi* and *V. fischeri* (see references 25 and 38 and references therein) and virulence in *V. harveyi* and *V.*

cholerae (25, 44). In both cases, it is thought that the involvement of multiple signals plays a role in fine-tuning the level and timing of responses to specific conditions (36, 39, 45, 61, 68, 80). In all these systems, the utilization of quorum-sensing systems that integrate multiple signals provides the cells the ability to modulate specific biological responses under a variety of conditions.

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