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Bacteria Possessing Two RelA/SpoT-Like Proteins Have Evolved a Specific Stringent Response Involving the Acyl Carrier Protein-SpoT Interaction[▽]

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Bacteria respond to nutritional stress by producing (p)ppGpp, which triggers a stringent response resulting in growth arrest and expression of resistance genes. In *Escherichia coli*, RelA produces (p)ppGpp upon amino acid starvation by detecting stalled ribosomes. The SpoT enzyme responds to various other types of starvation by unknown mechanisms. We previously described an interaction between SpoT and the central cofactor of lipid synthesis, acyl carrier protein (ACP), which is involved in detecting starvation signals in lipid metabolism and triggering SpoT-dependent (p)ppGpp accumulation. However, most bacteria possess a unique protein homologous to RelA/SpoT (Rsh) that is able to synthesize and degrade (p)ppGpp and is therefore more closely related to SpoT function. In this study, we asked if the ACP-SpoT interaction is specific for bacteria containing two RelA and SpoT enzymes or if it is a general feature that is conserved in Rsh enzymes. By testing various combinations of SpoT, RelA, and Rsh enzymes and ACPs of *E. coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Streptococcus pneumoniae*, we found that the interaction between (p)ppGpp synthases and ACP seemed to be restricted to SpoT proteins of bacteria containing the two RelA and SpoT proteins and to ACP proteins encoded by genes located in fatty acid synthesis operons. When Rsh enzymes from *B. subtilis* and *S. pneumoniae* are produced in *E. coli*, the behavior of these enzymes is different from the behavior of both RelA and SpoT proteins with respect to (p)ppGpp synthesis. This suggests that bacteria have evolved several different modes of (p)ppGpp regulation in order to respond to nutrient starvation.

All bacteria respond to sudden nutritional starvation through the stringent response. This response consists of the rapid shutdown of rRNA transcription and hence ribosome biogenesis by (p)ppGpp alarmone (4). Consequently, a whole set of stress response genes is activated. The level of (p)ppGpp in bacteria is controlled by enzymes belonging to the RelA/SpoT family. In *Escherichia coli*, there are two such enzymes, one that is able to synthesize (p)ppGpp (RelA) and one that is able to both synthesize and degrade (p)ppGpp (SpoT). RelA responds specifically to amino acid starvation, while SpoT responds to more diverse starvation events (carbon source, phosphate, and iron starvation) (28, 30, 34). Enzymes belonging to the RelA/SpoT family are organized in two domains; the N-terminal domain contains the enzymatic activities, and the C-terminal domain is involved in regulation of these enzymatic activities. The structure determined for the N-terminal catalytic domain of a bifunctional SpoT-like enzyme from *Streptococcus equisimilis* has provided clues about how the two opposite reactions, (p)ppGpp synthesis and degradation, might be regulated. The shift from one activity to the other may result from allosteric transition that is triggered by modifications in the C-terminal regulation domain (12). In *E. coli*, RelA is bound to the ribosomes and responds to stalling due to uncharged tRNA binding (31). Deletion of the C-terminal domain of RelA prevents interaction of this protein with the ribosome and leads to a constitutively active (p)ppGpp synthase activity, showing the importance of the regulation by the C-terminal domain (26). In contrast, the mechanism that results in SpoT-dependent (p)ppGpp accumulation has been a mystery for a long time. Recently, we characterized an interaction between SpoT and acyl carrier protein (ACP), a small protein acting as a cofactor in fatty acid and lipid metabolism (1). ACP interacts with the C-terminal domain of SpoT, and we isolated SpoT mutants that were not able to interact with ACP and that were also unable to respond to SpoT-specific starvation (1 and data not shown). We suggested that this interaction is involved in controlling SpoT activity and that perturbation of fatty acid metabolism may be detected by SpoT via ACP, which explains the SpoT-dependent response to fatty acid inhibition (27).

The presence of two separate and specialized SpoT and RelA paralogous proteins, such as the proteins in *E. coli*, is a feature shared only by beta- and gammaproteobacteria (21). In most bacteria, there is only one Rsh (Rel/Spo homolog) bifunctional enzyme that is able to degrade and synthesize (p)ppGpp. Because they possess both enzymatic activities, such enzymes are more closely related functionally to *E. coli* SpoT than to RelA (21). However, the situation is often complicated by the presence in the genomes of these bacteria of one or two additional genes coding for small putative proteins that contain only the (p)ppGpp-synthesizing enzymatic domain. This domain is referred to in the Pfam databank as the RelA/SpoT domain (PF04607) (7), but this designation is misleading because it refers solely to the (p)ppGpp synthase domain of SpoT, RelA, and Rsh proteins. Therefore, we call the

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proteins containing this domain RelP and RelQ, as proposed previously (15). It has been shown recently that in *Streptococcus mutans* and *Bacillus subtilis* these small proteins are expressed and function in the synthesis of (p)ppGpp and that they participate in regulation of the (p)ppGpp level in the cell (15, 22). Furthermore, a structure has been determined for the Q97QV1 protein of *Streptococcus pneumoniae* (PDB code 2be3) (M. E. Cuff, C. Hatzos, and A. Joachimiak, unpublished data) that is very similar to the structure of the (p)ppGpp synthase domain of the *S. equisimilis* Rsh protein (12).

It has been shown that when the Rsh protein of *S. equisimilis* is produced in *E. coli*, it does not behave like it does in *S. equisimilis*. In particular, *S. equisimilis* responds to amino acid starvation, whereas a Δ*relA* Δ*spoT E. coli* mutant expressing the *rsh* gene of *S. equisimilis* does not respond to these conditions (19). Furthermore, by using chimeric variants of RelA from *E. coli* and Rsh from *S. equisimilis*, the authors showed that the ability to respond to amino acid starvation in *E. coli* was conferred by the C-terminal domain of RelA (19). These results led to the suggestion that the specificity of protein-protein interactions may be responsible for different behaviors of the Rsh protein in the host and in *E. coli*. In this regard, we were interested in testing the host specificity of the interaction between ACP and SpoT that we observed (1). Because we demonstrated that this interaction was direct (it was also detected using yeast two-hybrid analyses) (1), we considered testing the interaction of heterologous Rsh and ACP proteins using bacterial two-hybrid analysis in *E. coli*.

In the present study, we asked if the link between stringent response and fatty acid metabolism that we documented is a specific feature of bacteria containing two proteins belonging to the RelA/SpoT family, concomitant with specialization of one of the proteins for fatty acid metabolism perturbation, or if it is a general feature shared by all bacteria. In a first attempt to answer this question, we studied the interactions between proteins belonging to the RelA/SpoT family and ACPs of bacteria containing various combinations of these proteins (*E. coli*, *P. aeruginosa*, *S. pneumoniae*, and *B. subtilis*) (Fig. 1). We found that the interaction between SpoT and ACP was restricted to bacteria containing two RelA and SpoT proteins and to ACPs encoded by genes in fatty acid synthesis operons. Furthermore, when produced in *E. coli*, the Rsh enzymes of *B. subtilis* and *S. pneumoniae* exhibited behaviors different from the behaviors of both the RelA and SpoT proteins with respect to (p)ppGpp synthesis. Our results suggest that bacteria have evolved several modes of (p)ppGpp regulation in order to respond to specific stresses.

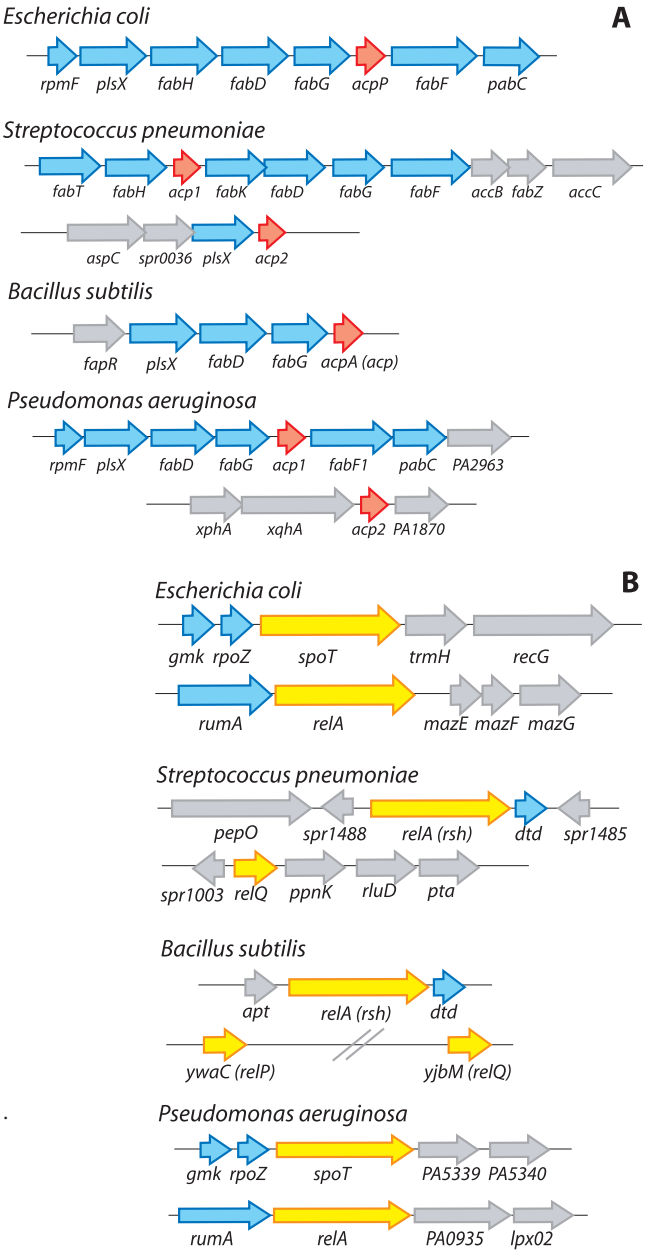

FIG. 1. Genetic context of *acp* genes (A) and *spoT*, *relA*, and *rsh* genes (B) in *E. coli*, *P. aeruginosa*, *S. pneumoniae*, and *B. subtilis*. *acp* genes are red, and *rel* and *spo* genes are yellow. In each panel, genes that are conserved in the genetic context are blue. The gene designations described previously or the gene annotations are indicated, and the designations that we used in the present study are indicated in parentheses. The designations for the *relA* genes of *S. pneumoniae* and *B. subtilis* are misleading, and these genes were designated rsh_{Spn} or rsh_{Bsu} in this study to avoid confusion. The *acpA* gene of *B. subtilis* is unique and is designated acp_{Bsu}

MATERIALS AND METHODS

Strains and media. All of the strains used are derivatives of *E. coli* K-12. The *cya* BTH101 strain (14) was used for the two-hybrid assay. The CF1652 (Δ*relA*), CF1693 [Δ*relA* Δ*spoT*; (p)ppGpp°], CF4941, and CF4943 (*spoT203*) strains (9, 34) were used to test the (p)ppGpp synthesis and degradation activities of proteins belonging to the RelA/SpoT family in complementation assays. The thermosensitive *acp* [*acpP*(Ts)] mutation from strain NRD53 (5) was transferred into MG1655 using P1 transduction. The resulting MG1655*acp*(Ts) strain was used to test ACP functionality. Finally, strain C600 (lab stock) was used for plasmid construction.

Cells were grown at 37°C in Luria-Bertani (LB) medium unless indicated otherwise (20). Plasmids were maintained with ampicillin (100 μg/ml) or kanamycin (50 μg/ml). Plates containing M9 minimal medium with glucose and serine-methionine-glycine (SMG) plates were prepared as described previously (20, 25).

Plasmid construction. For PCR gene amplification, the DNA matrix used was genomic DNA from *E. coli* MG1655, *P. aeruginosa* PAO1, *S. pneumoniae* R800, and *B. subtilis* 168; the only exception was the DNA for the $relA_{Pae}$ gene, for which we used the pUC19-$relA_{Pae}$ plasmid (29) as the matrix. The oligodeoxynucleotides used were purchased from Eurogentec SA and are shown in Table 1 along with the corresponding genes amplified and the UniProt identifi-

TABLE 1. Oligodeoxynucleotides used in this study and the corresponding cloned genes

Oligodeoxynucleotide	Sequence[a]	Gene	UniProt identification no.
Ebm321	CGAC**TCTAGA**ATTCATGAGCACCATCGAAGAACGCG	$acp1_{Pae}$	O54439
Ebm322	CTA**CTCGAG**AAGCTTATTGCTGGGGAGCAACGATGTAG		
Ebm372	CACC**GAATTC**ATGGACGACATCGAGACCAGAGTG	$acp2_{Pae}$	O52658
Ebm373	ACCG**ATCGAT**AAGCTTCAGGTCGGCACGGCTTCC		
Ebm346	CACC**GAATTC**ATGGCAGTATTTGAAAAAGTAC	$acp1_{Spn}$	P0A2W1
Ebm347	ACCG**CTCGAG**TTATTTTGCTTGCTCTTCAACGTAAG		
Ebm350	CACC**GAATTC**ATGACAGAAAAAGAAATTTTTG	$acp2_{Spn}$	Q8DRN2
Ebm351	ACCG**CTCGAG**CTATTTTCCTTGAATGATTTTAAC		
Ebm393	ACC**GAATTC**ATGGCAGACACATTAGAGCGTG	acp_{Bsu}	P80643
Ebm394	ACG**CTCGAG**TTATTGCTGGTTTTGTATGTAGTTC		
Ebm358	GAC**TCTAGA**ATTCTTGCCGGGCATAGACGCC	$spoT_{Pae}$	Q9HTM0
Ebm359	GACTCTAG**ATCGAT**CAGCTACGCAGGCGGGTG		
Ebm374	CACC**GAATTC**ATGGTACAGGTGAGAGCGCACC	$relA_{Pae}$	Q9I524
Ebm375	ACCG**ATCGAT**AAGCTTCAAGGCGTACGGTTGCGCC		
Ebm348	CGAC**TCTAGA**ATTCATGCCGAAAGAAGTGAATTTAAC	rsh_{Spn}	Q97PH2
Ebm349	ACCG**CTCGAG**CTAGCCGTTGGTCCGTTTGAC		
Ebm382	CACC**GAATTC**ATGGCGAACGAACAAGTA	rsh_{Bsu}	O54408
Ebm383	ACCG**CTCGAG**TTAGTTCATGACGCGGCG		
Ebm390	ACCG**AATTCA**TGACCTTAGAATGGGAAGAATTTC	$relQ_{Spn}$	Q97QV1
Ebm391	ACGC**TCGAGT**TACCTGTATTCTTCATCTGTATCG		
Ebm384	ACCG**AATTCA**TGGATTTATCTGTAACACATATGG	$relP_{Bsu}$	P39583
Ebm385	ACG**CTCGAG**TTAATCCACTTCTTTCTTAATCCCC		
Ebm386	ACC**GAATTC**ATGGATGACAAACAATGGGAGC	$relQ_{Bsu}$	O31611
Ebm387	ACG**CTCGAG**CTATTGTTGCTCGCTTCCTTTTTTC		
Ebm363	GATTATAAAGATGACGATGACAAG	T25FlagUp	
Ebm364	CTAGCTTGTCATCGTCATCTTTATAATCTGCA	T25FlagDown	
Ebm370	GGGATTATAAAGATGACGATGACAAG	T18FlagUp	
Ebm371	CTAGCTTGTCATCGTCATCTTTATAATCCCTGCA	T18FlagDown	

[a] Restriction sites used for construction are indicated by bold type.

cation numbers for the corresponding proteins. For all of the two-hybrid plasmid constructs, gene sequences were amplified by PCR using genomic DNA and oligonucleotides introducing EcoRI and XhoI sites (most constructs), XbaI and XhoI sites ($acp1_{Pae}$ and rsh_{Spn}), EcoRI and ClaI sites ($acp2_{Pae}$ and $relA_{Pae}$), or XbaI and ClaI sites ($spoT_{Pae}$). The DNA fragments were then ligated into plasmids pT18 (pEB355) and pT25 (pEB354) (10) or into plasmids pT18Flag (pEB1030) and pT25Flag (pEB1029) (2) using the same sites.

Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrotransfer onto nitrocellulose membranes, and Western blot analysis were performed as previously described (11). Monoclonal anti-Flag M2 antibodies were purchased from Sigma. Anti-RelA serum was a kind gift from M. Cashel. The T18 domain was detected by far Western blotting using biotinylated calmodulin as described elsewhere (2).

Bacterial two-hybrid assay. We used the adenylate cyclase-based two-hybrid technique (14). Pairs of the proteins to be tested were fused to the T18 and T25 catalytic domains of adenylate cyclase using plasmids pT18 and pT25 or pT18Flag and pT25Flag. After cotransformation of the BTH101 strain with the two plasmids expressing the fusions, selection plates were incubated at 30°C for 48 h. Three milliliters of LB medium supplemented with ampicillin, kanamycin, and 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was inoculated and incubated at 30°C for 18 h, and then β-galactosidase assays were performed as previously described (1).

Screening for (p)ppGpp synthesis. (p)ppGpp was measured as described by Cashel (3). In brief, fresh cells were transferred directly from LB medium plates into morpholinepropanesulfonic acid (MOPS) medium containing 2% glucose and 0.2 mg/ml serine hydroxamate (SHX) and transferred into the same medium containing 10 μCi/ml of ^{32}P. After 5, 10, and 15 min, aliquots were removed and mixed with an equal volume of 16 M formic acid on ice. Five microliters of each of the formic acid extracts was chromatographed in one dimension on polyethyleneimine cellulose thin-layer chromatography plates (20 by 10 cm; J. T. Baker). The thin-layer chromatography plates were then exposed to film for autoradiography.

RESULTS

Cloning of the *spoT*, *relA*, *rsh*,and *acp* genes and functionality studies. In *E. coli*, there is only one *acpP* gene, which is located in the fatty acid synthesis operon (Fig. 1A). In *P. aeruginosa*, *S. pneumoniae*, and *B. subtilis*, a corresponding *acp* gene that we designated *acp1* is present in equivalent operons involved in fatty acid synthesis (Fig. 1A and Table 1). However, *P. aeruginosa* and *S. pneumoniae* both contain an additional

acp gene with an unknown function (designated *acp2*), which is located in chromosomal regions not related to fatty acid synthesis (Fig. 1A). Interestingly, in the genome of *S. pneumoniae*, *acp2* is located next to *plsX*, a gene coding for an enzyme involved in the initial step of phospholipid synthesis that is often found in the fatty acid synthesis operon, especially in *E. coli* (16) (Fig. 1A). *E. coli* and *P. aeruginosa* contain two genes belonging to the *relA*/*spoT* family, one corresponding to *spoT* [(p)ppGpp degradation and synthesis] and the other corresponding to *relA* [(p)ppGpp synthesis only]. In *S. pneumoniae* and *B. subtilis*, there is only one full-length *rsh* gene, and it has both (p)ppGpp synthesis and degradation domains (Fig. 1B).

Two-hybrid plasmids were constructed to study the interactions between ACP and the SpoT, RelA, and Rsh proteins of *E. coli*, *P. aeruginosa*, *S. pneumoniae*, and *B. subtilis* and also to assess the functionality of these proteins in *E. coli*. Sequences were amplified from genomic DNA of *E. coli* MG1655, *P. aeruginosa* PAO1, *S. pneumoniae* R800, and *B. subtilis* 168 and inserted into the pT18 and pT25 plasmids (*spoT*, *relA*, and *rsh* genes) or into the modified pT18Flag two-hybrid plasmid (*acp* genes) (see Materials and Methods). In order to verify that fusion with the two hybrid domains did not destabilize the proteins, we examined whether the expression and function in *E. coli* were correct.

(i) Expression of recombinant Flag-ACPs and RelA, SpoT, and Rsh proteins. In order to monitor production of the ACP recombinant proteins, we used the pT18Flag two-hybrid plasmid that contains an additional Flag epitope enabling detection by the monoclonal anti-Flag M2 antibody (2). We showed that all T18Flag-ACP recombinant proteins were expressed at comparable levels in *E. coli* (Fig. 2A).

In order to monitor production of the SpoT, RelA, and Rsh recombinant proteins, we performed sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by far Western blotting with biotinylated calmodulin, in order to detect the T18 domain (2). We observed that each T18-SpoT, -RelA, or -Rsh fusion protein was correctly produced and that comparable amounts were produced (Fig. 2B). In parallel, we also performed Western blotting using anti-RelA or anti-SpoT antisera prepared for the *E. coli* RelA and SpoT proteins. However, apart from the $RelA_{Eco}$ and $SpoT_{Eco}$ proteins, the only proteins that we were able to detect were the $RelA_{Pae}$ and Rsh_{Spn} recombinant proteins using the anti-RelA antiserum, showing the variable specificity of the antibodies (Fig. 2B and data not shown).

(ii) Functionality of the recombinant ACPs in *E. coli*. It is possible to test the functionality in *E. coli* of recombinant *acp* genes using an *acpP*(Ts) mutant obtained recently in the laboratory of J. E. Cronan (5, 6). pT18Flag-$acpP_{Eco}$ was able to functionally replace the *acpP*(Ts) allele at 42°C in strain MG1655*acpP*(Ts) (Fig. 3). This showed that fusion of the T18Flag domain at the N-terminal end of ACP_{Eco} did not impair the function of ACP_{Eco}. Therefore, we were able to test the functionality in *E. coli* of the other ACP recombinant proteins. The MG1655*acpP*(Ts) strain was transformed by using a series of pT18Flag constructs. In addition to pT18Flag-$acpP_{Eco}$, the pT18Flag-$acp1_{Pae}$, pT18Flag-$acp1_{Spn}$, and pT18Flag-acp_{Bsu} plasmids restored the growth of the MG1655*acpP*(Ts) strain at 42°C (Fig. 3). It was shown previously that the acp_{Bsu} gene compensated for the *acpP*(Ts) mutation in *E. coli* (6). This demonstrated again that fusion with the two-hybrid domain did not alter the function of the protein. In contrast, plasmids pT18Flag-$acp2_{Pae}$ and pT18Flag-$acp2_{Spn}$ did not complement the MG1655*acpP*(Ts) strain (Fig. 3).

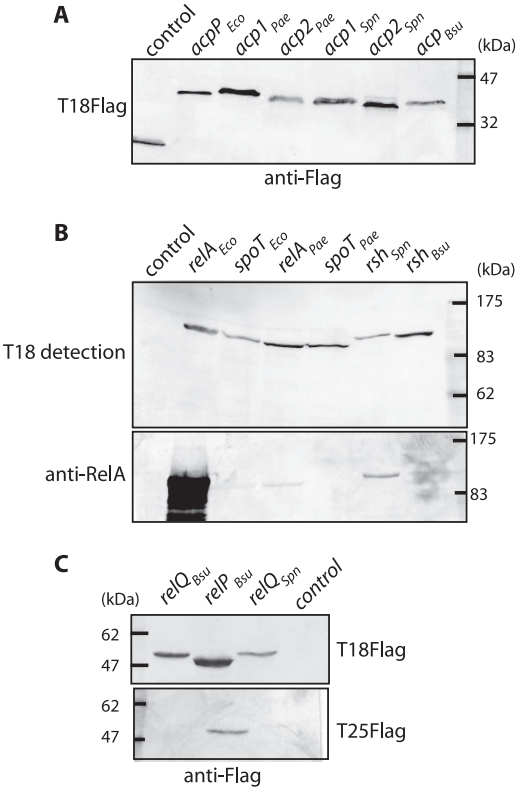

FIG. 2. Expression of the recombinant proteins. (A) T18Flag-ACPs. Strain C600 was transformed with the pT18Flag series of plasmids containing the indicated *acp* genes. After induction for 3 h with 0.5 mM IPTG in LB medium at 37°C, the recombinant proteins were detected by Western blotting using anti-Flag M2 antibody. (B) T18-SpoT, -RelA, and -Rsh proteins. Strain C600 was transformed with the pT18Flag series of plasmids containing the indicated *relA*, *spoT*, and *rsh* genes. After induction for 3 h with 0.5 mM IPTG in LB medium at 37°C, the recombinant proteins were detected by far Western blotting with biotinylated calmodulin and by Western blotting using anti-RelA antibodies. (C) T18Flag- and T25Flag-RelP-like and -RelQ-like proteins. Strain C600 was transformed with the pT18Flag and pT25Flag series of plasmids containing the indicated genes. After induction for 3 h with 0.5 mM IPTG in LB medium at 37°C, the recombinant proteins were detected by Western blotting using anti-Flag M2 antibody.

We concluded that $ACP1_{Pae}$, $ACP1_{Spn}$, and ACP_{Bsu} are functional equivalents of ACP in *E. coli* and that $ACP2_{Pae}$ and $ACP2_{Spn}$ may have other functions in *P. aeruginosa* and *S. pneumoniae*.

(iii) Functionality of the recombinant SpoT, RelA, and Rsh proteins in *E. coli*. In order to test the functionalities and (p)ppGpp synthesis or degradation activities of the two-hybrid SpoT, RelA, and Rsh proteins, we used well-described complementation tests with *relA* and *spoT* mutants of *E. coli*. The Δ*relA* Δ*spoT* CF1693 strain is not able to grow on minimal media without amino acids due to the complete absence of (p)ppGpp (34). The Δ*relA* CF1652 strain is not able to grow when it is subjected to isoleucine starvation on SMG plates due

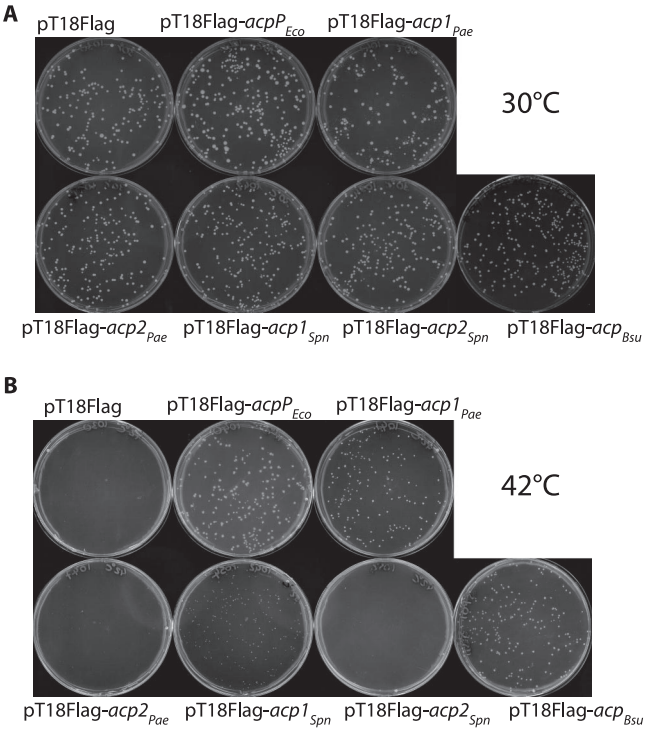

FIG. 3. Functionality of the recombinant ACPs. The MG1655*acp*(Ts) strain was transformed with the indicated pT18Flag-*acp* plasmids, plated on LB medium plates containing ampicillin, and incubated at 30°C for 3 days (A) or at 42°C for 36 h (B).

to the small amount of (p)ppGpp present (34). Finally, the Δ*relA spoT203* CF4943 strain contains high basal levels of (p)ppGpp due to a *spoT* defect in (p)ppGpp degradation, which results in a slow-growth phenotype on rich medium (9).

It was impossible to transform the CF1693 (Δ*relA* Δ*spoT*) and CF4943 (Δ*relA spoT203*) strains with pT18-*relA*$_{Eco}$, while pT18-*relA*$_{Eco}$ did restore growth of CF1652 (Δ*relA*) on SMG plates (data not shown). This demonstrated that T18-RelA$_{Eco}$ was able to synthesize high levels of (p)ppGpp, which is toxic when the *spoT* product is not present to degrade it. Inversely, pT18-*spoT*$_{Eco}$ complemented the phenotypes of the CF1693 and CF4943 strains but not the phenotype of CF1652, as described previously (1) (Fig. 4 and data not shown). This demonstrated that T18-SpoT$_{Eco}$ was able to both synthesize and degrade (p)ppGpp. These data showed that the two-hybrid recombinant constructs behaved like untagged clones described previously (8) and that we could use these constructs for functional studies. The pT18-*spoT*$_{Pae}$ plasmid behaved exactly like pT18-*spoT*$_{Eco}$, demonstrating that the T18-SpoT$_{Pae}$ recombinant protein was also able to synthesize and degrade (p)ppGpp (Fig. 4). As observed for pT18-*relA*$_{Eco}$, it was not possible to transform the CF1693 and CF4943 strains with pT18-*relA*$_{Pae}$ (we obtained only very small colonies on LB medium plates, which were not viable in liquid medium), showing that T18-RelA$_{Pae}$ synthesized (p)ppGpp. However, the (p)ppGpp level was not high enough to complement CF1652 for growth on SMG plates (data not shown). pT18-*rsh*$_{Spn}$ and pT18-*rsh*$_{Bsu}$ behaved like pT18-*spoT*$_{Eco}$ with respect to complementation of the CF1693 and CF4943 strains (Fig. 4). This demonstrated that these constructs were able to synthesize and degrade (p)ppGpp. However, when growth in liquid minimal medium without amino acids was examined, there was a clear difference in growth rate between CF1693 transformed with pT18-*spoT*$_{Eco}$ or pT18-*spoT*$_{Pae}$ on the one hand and CF1693 transformed with pT18-*rsh*$_{Bsu}$ or pT18-*spoT*$_{Spn}$ on the other hand (data not shown). The latter strains grew very poorly. Taking into account the central and subtle role of (p)ppGpp in growth control, even if all the Rsh recombinant proteins were functional, some species-related specificity might explain the quantitative differences (see below). The results of these phenotype tests are summarized in Table 2.

Interactions between ACPs and the RelA, SpoT, and Rsh proteins. We systematically screened the interactions between the SpoT, RelA, and Rsh proteins and ACPs in *E. coli* using

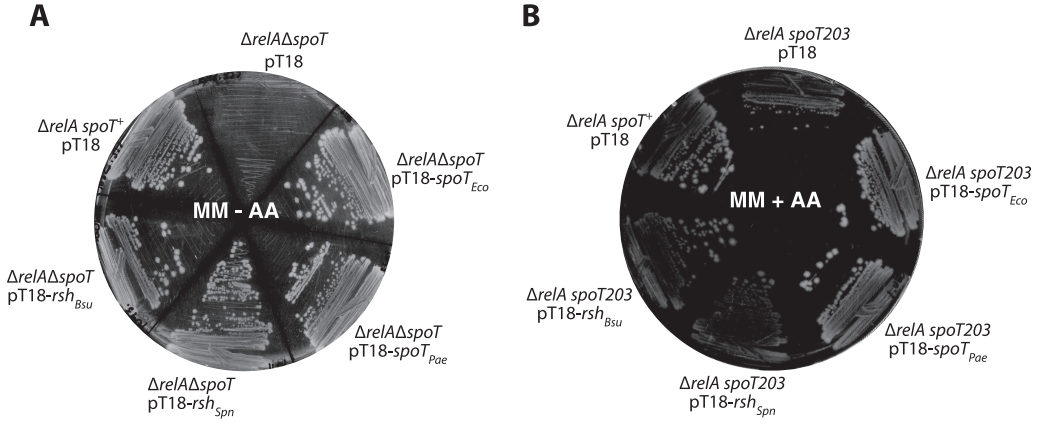

FIG. 4. Functionality of the recombinant SpoT, RelA, and Rsh proteins. (A) Complementation of CF1693. Strain CF1693 (Δ*relA* Δ*spoT*) was transformed with the indicated plasmids. Clones selected on LB agar plates containing ampicillin were replicated on M9 minimal agar plates containing ampicillin without amino acids (MM-AA) and incubated for 48 h at 37°C together with strain CF1652 (Δ*relA spoT*$^+$) transformed with pT18 as a positive control. (B) Complementation of CF4943. Strain CF4943 (Δ*relA spoT203*) was transformed with the indicated plasmids. Clones selected on LB agar plates containing ampicillin were replicated on M9 minimal agar plates containing ampicillin supplemented with amino acids (40 μg/ml each) (MM+AA) and incubated for 24 h at 37°C together with strain CF4941 (Δ*relA spoT*$^+$) transformed with pT18 as a positive control.

TABLE 2. Summary of the phenotypes observed for reporter strains

Plasmid	Growth of CF1693 on M9 minimal medium[a]	Complementation of slow-growth phenotype of CF4943 on LB medium[b]	Detection of ppGpp in CF1652[c]	Deduced activity
pT18 plasmids with *rel*/*spo* genes				
Empty	−	−	−	
$relA_{Eco}$	tox	tox	+	High ppGpp synthesis
$spoT_{Eco}$	+	+	−	Balanced activities
$relA_{Pae}$	tox	tox	+/−	ppGpp synthesis
$spoT_{Pae}$	+	+	−	Balanced activities
rsh_{Spn}	+	+	−	Balanced activities
rsh_{Bsu}	+	+	−	Balanced activities
pT18Flag plasmids with *relP* or *relQ* genes				
$relQ_{Spn}$	tox	tox	+/−	ppGpp synthesis
$relP_{Bsu}$	tox	tox	+	High ppGpp synthesis
$relQ_{Bsu}$	tox	tox	+/−	ppGpp synthesis

[a] +, growth of CF1693 transformed with the indicated plasmid on M9 minimal medium containing glucose; −, no growth; tox, toxic (no transformants of the corresponding strain or only small nonviable colonies).
[b] +, complementation of the slow-growth phenotype of CF4943; −, no complementation; tox, toxic (no transformants of the corresponding strain or only small nonviable colonies).
[c] +, ppGpp detected in CF1652 using the rapid screening assay; −, ppGpp not detected in CF1652 using the rapid screening assay.

the bacterial two-hybrid technique (14). We first verified that use of the new pT18Flag construct did not interfere with detection of the SpoT-ACP interaction that we observed previously when the two-hybrid technique was used (1). Indeed, we detected an interaction between T25-$SpoT_{Eco}$ and T18Flag-ACP_{Eco} identical to the interaction between T25-$SpoT_{Eco}$ and T18-ACP_{Eco} (Fig. 5). Moreover, we detected homodimerization of all six SpoT, RelA, and Rsh proteins studied (data not shown), indicating that the T25 fused proteins were correctly expressed. We then tested each SpoT, RelA, or Rsh protein to examine its interaction with the ACP(s) from the same organism. $SpoT_{Pae}$ interacted with $ACP1_{Pae}$ and $ACP2_{Pae}$, but the signal was weaker for $ACP2_{Pae}$ (150 Miller units compared to 600 Miller units) (Fig. 5). Therefore, the interaction between SpoT and ACP is conserved in *P. aeruginosa* and is relatively specific for the *acp1* gene located in the operon for fatty acid synthesis. In contrast, we were not able to detect any interaction between the Rsh enzymes from *S. pneumoniae* and *B. subtilis* and any of their ACPs (Fig. 5). Therefore, we concluded that the interaction with ACP is not conserved for Rsh proteins of *S. pneumoniae* and *B. subtilis*.

	T25 $RelA_{Eco}$	T25 $SpoT_{Eco}$	T25 $RelA_{Pae}$	T25 $SpoT_{Pae}$	T25 Rsh_{Spn}	T25 Rsh_{Bsu}
T18Flag ACP_{Eco}	-	700	-	300	-	-
T18Flag $ACP1_{Pae}$	-	600	-	600	ND	ND
T18Flag $ACP2_{Pae}$	-	200	-	150	ND	ND
T18Flag $ACP1_{Spn}$	ND	150	ND	-	-	ND
T18Flag $ACP2_{Spn}$	ND	-	ND	-	-	ND
T18Flag ACP_{Bsu}	ND	550	ND	-	ND	-

FIG. 5. Interactions between ACP and the RelA, SpoT, and Rsh proteins determined by bacterial two-hybrid analysis. Interactions between the ACPs fused to the T18Flag domain and the RelA, SpoT, and Rsh proteins fused to the T25 domain were assayed by the two-hybrid method as described in Materials and Methods. Black cells, strong interaction; gray cells, interaction; −, no interaction; ND, not determined. The β-galactosidase activity values (in Miller units) are indicated for the significant interactions.

Interestingly, when we tested the interaction of $SpoT_{Eco}$ with the ACPs of the other species, we detected interactions with $ACP1_{Pae}$ and ACP_{Bsu} and a weaker interaction with $ACP1_{Spn}$ (Fig. 5). The same results were obtained for $SpoT_{Pae}$. Therefore, the lack of interactions of the Rsh proteins from *S. pneumoniae* and *B. subtilis* with their ACPs is due to distinct features of the Rsh and SpoT proteins and not to distinct features of the ACPs.

SpoT-, RelA-, and Rsh-dependent accumulation of (p)ppGpp in *E. coli*. The complementation tests performed with minimal media and the CF1693 reporter strain demonstrated that Rsh_{Spn} and Rsh_{Bsu} could synthesize enough (p)ppGpp to restore amino acid synthesis (Fig. 4A). In order to screen for the ability of the SpoT, RelA, and Rsh proteins to respond to amino acid starvation, we performed a (p)ppGpp synthesis screening assay using minimal medium with SHX (3) and the CF1652 strain transformed with plasmids belonging to the pT18 plasmid series. Under these conditions (Δ*relA* $spoT^+$ context), only T18-$RelA_{Eco}$ and T18-$RelA_{Pae}$ provoked clear (p)ppGpp accumulation, demonstrating that there was (p)ppGpp synthesis activity that was greater than the endogenous $SpoT_{Eco}$ degradation activity present in the CF1652 strain (Fig. 6A). T18-$SpoT_{Eco}$, T18-$SpoT_{Pae}$, T18-Rsh_{Spn}, or T18-Rsh_{Bsu} production in CF1652 was not able to provoke accumulation of (p)ppGpp in response to SHX in this context (data not shown and Fig. 6A).

(p)ppGpp synthesis screening using minimal medium with SHX was also performed with the CF1693 strain (Δ*spoT* Δ*relA* context). In this experiment, (p)ppGpp accumulation was detected with Rsh_{Bsu} and Rsh_{Spn} but not with $SpoT_{Eco}$ and $SpoT_{Pae}$ (Fig. 6B). This indicated that the Rsh_{Spn} and Rsh_{Bsu} proteins, in contrast to $SpoT_{Eco}$ and $SpoT_{Pae}$, were able to respond to amino acid starvation caused by SHX. However, the response

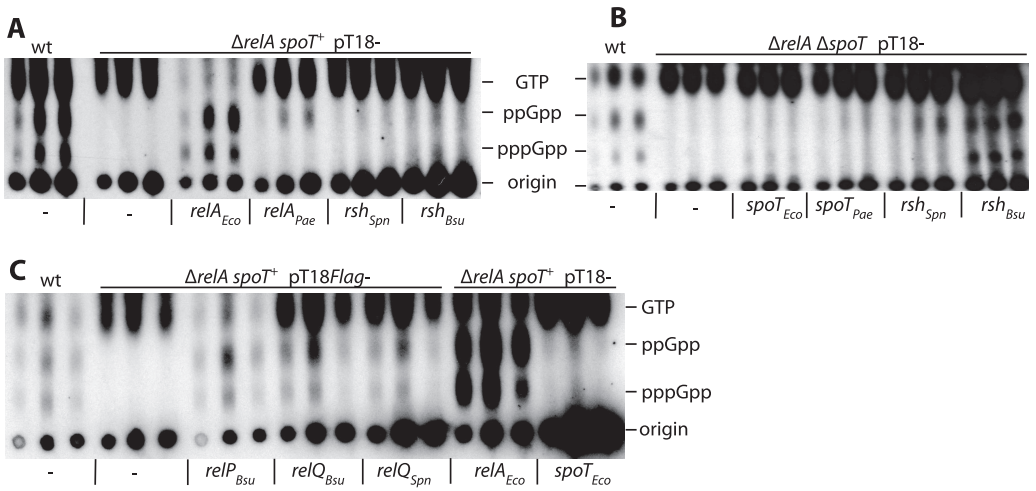

FIG. 6. Response to amino acid starvation determined by a rapid (p)ppGpp synthesis assay using SHX medium. Labeling was performed as described in Materials and Methods. Autoradiographs of the thin-layer chromatography plates are shown. In each case, a control experiment with the wild-type MG1655 strain transformed with pT18 was performed (wt). For each experiment, the results obtained at three time points (5, 10, and 15 min) are shown. (A) Strain CF1652 (Δ*relA spoT*$^+$) transformed with the pT18-*relA*$_{Eco}$, pT18-*relA*$_{Pae}$, pT18-*rsh*$_{Spn}$, and pT18-*rsh*$_{Bsu}$ plasmids. (B) Strain CF1693 (Δ*relA* Δ*spoT*) transformed with the pT18-*spoT*$_{Eco}$, pT18-*spoT*$_{Pae}$, pT18-*rsh*$_{Spn}$, and pT18-*rsh*$_{Bsu}$ plasmids. (C) Strain CF1652 (Δ*relA spoT*$^+$) transformed with the pT18-*relQ*$_{Bsu}$, pT18-*relP*$_{Bsu}$, pT18-*relQ*$_{Spn}$, pT18-*relA*$_{Eco}$, and pT18-*spoT*$_{Eco}$ plasmids as controls.

was lower than the response observed with RelA$_{Eco}$ or RelA$_{Pae}$. Indeed, RelA$_{Eco}$ or RelA$_{Pae}$ (p)ppGpp synthesis activity overcame SpoT$_{Eco}$ endogenous deacylation activity, whereas Rsh$_{Spn}$ or Rsh$_{Bsu}$ (p)ppGpp synthesis activity did not (Fig. 6A).

Activities of the RelP and RelQ proteins from *S. pneumoniae* and *B. subtilis*. *S. pneumoniae* and *B. subtilis* possess additional small proteins containing a (p)ppGpp synthesis domain that could participate in the nutritional stress response mechanism in these bacteria. Only one such protein has been found in *S. pneumoniae* (UniProt identification no. Q97QV1), and it corresponds to RelQ of *S. mutans* (15) (75% sequence identity). RelQ$_{Spn}$ structure has been solved (PDB identification no. 2be3) (Cuff et al., unpublished data). In *B. subtilis*, YwaC and YjbM are 31 and 52% identical to RelP and RelQ of *S. mutans*, respectively. The attribution is not as clear for RelP$_{Bsu}$ (YwaC), but we took this nomenclature based on comparison with the closely related RelP and RelQ proteins identified in *Bacillus anthracis* (15), which is also consistent with the two clades (SAS1 and SAS2) proposed by other authors (22).

We cloned the genes coding for these proteins in the pT18Flag and pT25Flag vectors as described above for *acp* genes. We verified that the recombinant proteins were correctly expressed using anti-Flag antibodies (Fig. 2C). The amount of protein was much smaller in the pT25Flag series than in the pT18Flag series due to the low copy number of the pT25Flag plasmid. We used the *E. coli* reporter strains described above to determine the activities of the recombinant proteins. The three proteins produced (p)ppGpp, as demonstrated by the fact that viable transformants of CF1693 and CF4943 could not be obtained (Table 2 and data not shown). (p)ppGpp synthesis was further proven by performing a rapid (p)ppGpp synthesis screening assay. The CF1652 strain transformed with pT18-*relP*$_{Bsu}$, pT18-*relQ*$_{Bsu}$, or pT18-*relQ*$_{Spn}$ accumulated (p)ppGpp in the presence of SHX (Fig. 6C). RelP$_{Bsu}$ produced a larger quantity of (p)ppGpp than RelQ$_{Bsu}$ and RelQ$_{Spn}$, comparable to the production data for RelA$_{Eco}$ if the (p)ppGpp/GTP ratio is considered. RelQ$_{Bsu}$ and RelQ$_{Spn}$ did not produce as much (p)ppGpp as RelA$_{Eco}$, but they produced much more (p)ppGpp than SpoT$_{Eco}$ (Fig. 6C). These phenotypes are summarized in Table 2. They are strong indications that the RelP$_{Bsu}$, RelQ$_{Bsu}$, and RelQ$_{Spn}$ proteins are functional in their original organisms (*S. pneumoniae* and *B. subtilis*). During the course of this work, the (p)ppGpp synthesis activity of RelP$_{Bsu}$ and RelQ$_{Bsu}$ in *B. subtilis* was reported (22). The results that we report here are in accordance with the results of the study of Nanamiya et al. (22).

Using the two-hybrid technique, we detected no interaction between RelP$_{Bsu}$, RelQ$_{Bsu}$, or RelQ$_{Spn}$ and any of the ACPs (data not shown). This result was expected since the RelP$_{Bsu}$, RelQ$_{Bsu}$, and RelQ$_{Spn}$ proteins do not contain the TGS domain that we demonstrated previously to be required for ACP binding (1).

DISCUSSION

In the present study, we showed that the SpoT-ACP interaction found previously in *E. coli* also occurs with the *P. aeruginosa* SpoT and ACP proteins. However, no direct interactions involving the Rsh proteins of *S. pneumoniae* and *B. subtilis* and their cognate ACPs were detected. This finding correlates with the fact that despite their ability to produce and degrade (p)ppGpp in *E. coli*, the Rsh enzymes of *S. pneumoniae* and *B. subtilis* did not behave like the SpoT$_{Eco}$ enzyme. SpoT proteins of *E. coli* and *P. aeruginosa* interacted with the ACPs encoded by genes located in fatty acid synthesis operons and not with ACP homologs encoded in other genetic contexts, reinforcing our hypothesis that there is a link between a SpoT-dependent response and fatty acid metabolism.

Specificity of the interaction between SpoT proteins and ACPs. One may wonder if the inability to detect an interaction between Rsh$_{Bsu}$ or Rsh$_{Spn}$ and ACPs was a consequence of the fact that these interactions were studied in *E. coli* and not in

the host organisms. Furthermore, the interactions between hybrid proteins were detected with potential competition of the endogenous ACP, RelA, and SpoT *E. coli* proteins present in the strain used for the assay. However, several factors indicate that the approach used is reliable: (i) we ascertained that the ACP-SpoT$_{Eco}$ interaction was direct, (ii) we showed that the expression and functionality of the heterologous proteins produced in *E. coli* were correct, (iii) ACP$_{Bsu}$ and ACP1$_{Spn}$ were able to interact with SpoT$_{Eco}$, and (iv) the presence of endogenous ACP, RelA, and SpoT did not prevent detection of the interaction between ACP and SpoT of *E. coli*.

Rsh and SpoT proteins are homologous proteins that have very similar sequences (40% identity and 60% similarity for SpoT$_{Eco}$ and Rsh$_{Bsu}$, for example). Yet Rsh proteins did not interact with ACP. Even though SpoT$_{Eco}$ and SpoT$_{Pae}$ are more closely related (50% identity and 70% similarity), it is still difficult to understand what the determinant of specificity for the interaction of ACP with SpoT and not with the RelA and Rsh proteins is, and this is intriguing. In a previous study in which we compared SpoT$_{Eco}$ and RelA$_{Eco}$, we showed that the specificity of the interaction with ACP seemed to be determined by the N-terminal domain of the protein (1). However, the Rsh proteins are more closely related to SpoT$_{Eco}$ than RelA$_{Eco}$ is, especially when the N-terminal and TGS domains are compared, because they possess the same N-terminal (p)ppGpp synthesis and degradation catalytic domains. Therefore, the specificity of the interaction with ACP might be subtler. One specific biochemical feature of SpoT proteins that stands out is their basic pIs (pI 8.9 and 9.1 for SpoT$_{Eco}$ and SpoT$_{Pae}$, respectively), compared to the pIs of the RelA and Rsh proteins, which are more neutral pI (pI 6.3 to 7.6). It is well known that the mechanism by which ACP interacts with its numerous partner enzymes in fatty acid synthesis relies on electrostatic complementarity between negative and positive surfaces (35). Therefore, it can be proposed that SpoT proteins may have evolved toward a basic pI in order to favor the interaction with the acidic ACP.

SpoT$_{Eco}$ interacts with ACPs of the four species studied. When two ACPs are present (in *P. aeruginosa* and *S. pneumoniae*), the interaction is significantly stronger with the ACP1 protein encoded by the fatty acid synthesis operon than with the ACP2 protein having an unknown function, which correlates with the similarity of the different ACPs to ACP$_{Eco}$. SpoT$_{Pae}$ also interacts with ACP$_{Eco}$ and ACP1$_{Pae}$ more than it interacts with ACP2$_{Pae}$. Therefore, even if there is no species specificity for the ACP interaction, there is specificity for ACPs encoded by the fatty acid synthesis operon. It is noteworthy that these ACPs correspond to those that are able to complement the MG1655*acpP*(Ts) strain (Fig. 3).

SpoT, RelA, and Rsh enzyme activities in *E. coli*. Our results suggest that the SpoT$_{Pae}$ and RelA$_{Pae}$ proteins function like SpoT$_{Eco}$ and RelA$_{Eco}$, respectively. Indeed, only SpoT$_{Pae}$ interacts with ACP, as does SpoT$_{Eco}$. Furthermore, SpoT$_{Pae}$ behaved like SpoT$_{Eco}$ with respect to phenotypic complementation of reporter strains or (p)ppGpp production (Fig. 4 and 6). It was not possible to transform Δ*relA* Δ*spoT* or Δ*relA spoT203* strains with plasmids expressing *relA*$_{Pae}$ (as observed with *relA*$_{Eco}$). However, in contrast to *relA*$_{Eco}$, *relA*$_{Pae}$ did not complement the CF1652 (Δ*relA*) strain for growth on SMG (data not shown). Figure 6A shows that the (p)ppGpp synthesis in response to amino acid starvation of CF1652/*relA*$_{Pae}$ was indeed much weaker than the (p)ppGpp synthesis in response to amino acid starvation of CF1652/*relA*$_{Eco}$. Yet (p)ppGpp accumulation was visible in this *spoT*$^+$ background, demonstrating that the *relA*$_{Pae}$ product has a RelA-like activity.

Transformants of the (p)ppGpp° CF1693 strain containing *rsh*$_{Spn}$ or *rsh*$_{Bsu}$ were able to grow on minimal medium without amino acids, indicating that these genes encode a source of (p)ppGpp synthesis. We found that production of Rsh$_{Spn}$ and Rsh$_{Bsu}$ in *E. coli* CF1652 (Δ*relA*) did not result in (p)ppGpp accumulation in response to amino acid starvation and behaved like SpoT$_{Eco}$ in this regard (Fig. 6A). The same result was obtained previously with Rsh of *S. equisimilis* (17). However, *S. equisimilis*, *S. mutans*, and *B. subtilis* are organisms that are known to trigger (p)ppGpp accumulation in response to amino acid starvation (15, 18, 32), but in these organisms the accumulation does not have to overcome the hydrolase activity of a distinct enzyme and the increase in the synthetic activity is certainly accompanied by inhibition of the hydrolase activity exhibited by the same protein, as the structure of the N-terminal domain of *S. equisimilis* Rsh suggests (12). Indeed, when the protein is produced in the Δ*relA* Δ*spoT* strain, the response to amino starvation by Rsh$_{Bsu}$ (and to a lesser extent Rsh$_{Spn}$) can be detected, which is not the case for SpoT$_{Eco}$ and SpoT$_{Pae}$ (or the response is much lower) (Fig. 6B). This finding is comparable to what has been observed with Rsh of *S. mutans* (23).

The current model for the mechanism of regulation of RelA/SpoT family enzymes proposes that the C-terminal domain, by interacting with the N-terminal domain, maintains the enzyme in a hydrolase-dominant state in the case of the SpoT and Rsh proteins or in a low-synthesis state in the case of RelA (19). The C-terminal domain may be regulated by ligand binding, triggering its dissociation from the N-terminal domain and inhibition of the hydrolase activity. The nature of the ligand remains an open question. We propose that ACP is a regulating protein for SpoT-like proteins (1). It has been shown that the small GTPase CgtA also interacts with SpoT and that the interaction maintains SpoT in a hydrolysis-dominant state (13, 24, 33). In the case of RelA, the interaction of the C-terminal domain with the ribosome is the major regulating mechanism (31). It is possible that the differences observed in the activities of the SpoT, RelA, and Rsh enzymes expressed in *E. coli* result from the absence of such interactions naturally occurring in the native hosts and that this reflects the host specificity of protein-protein interactions. Supporting this hypothesis, it has been shown that replacing the C-terminal domain of Rsh of *S. equisimilis* with the C-terminal domain of RelA$_{Eco}$ restores the response to amino acid starvation in a (p)ppGpp° *E. coli* strain expressing the Rsh chimera (19).

Diversity of (p)ppGpp control mechanisms in bacteria. The presence of the *spoT* and *relA* genes in the bacterial genome is restricted to beta- and gammaproteobacteria (21). These organisms are gram-negative enterobacteria and are often causative agents of diseases. Their lifestyles may be the reason for the evolution of two genes belonging to the RelA/SpoT family. The link that we found between SpoT and lipid metabolism may indicate that specificity is related to the cellular envelope, which consists of two membranes. However, gram-negative bacteria are not restricted to the beta- and gammaproteobac-

terial subdivisions, and other gram-negative bacteria, such as cyanobacteria, contain only one Rsh protein. In gram-positive *Firmicutes* having only one Rsh, control of the (p)ppGpp levels is driven by the Rsh enzyme and one or two RelP-like and RelQ-like proteins (15, 22). Therefore, there are diverse modes of control of (p)ppGpp in bacteria that mediate the starvation stress response, as well as tune the bacterial growth rate to the nutritional quality of the environment. During evolution, different types of proteins may have specialized, with various mechanisms of (p)ppGpp regulation developing in response to specific nutritional stresses. For example, the ACP-SpoT interaction may correspond to a specific response when lipid metabolism is perturbed (1). Another example is the *relP* (*ywaC*) gene of *B. subtilis*, which is induced in response to alkaline stress (22). In the latter example, the C-terminal domain that normally plays a regulatory role in the RelA, SpoT, or Rsh proteins is replaced by regulation at the genetic level. To better understand these different mechanisms of regulation, it is important to clarify the types of starvation signals that are present in different bacteria and the genes that are involved in the specific responses.

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