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# Identification of *Legionella pneumophila* Effectors Regulated by the LetAS-RsmYZ-CsrA Regulatory Cascade, Many of Which Modulate Vesicular Trafficking

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***Legionella pneumophila*, the causative agent of Legionnaires' disease, is an intracellular human pathogen that utilizes the Icm/Dot type IVB secretion system to translocate a large repertoire of effectors into host cells. To find coregulated effectors, we performed a bioinformatic genomic screen with the aim of identifying effector-encoding genes containing putative CsrA regulatory elements. The regulation of these genes by the LetAS-RsmYZ-CsrA regulatory cascade was experimentally validated by examining their levels of expression in deletion mutants of relevant regulators and by site-directed mutagenesis of the putative CsrA sites. These analyses resulted in the identification of 26 effector-encoding genes regulated by the LetAS-RsmYZ-CsrA regulatory cascade, all of which were expressed at higher levels during the stationary phase. To determine if any of these effectors is involved in modulating the secretory pathway, they were overexpressed in wild-type yeast as well as in a yeast *sec22* deletion mutant, which encodes an R-SNARE that participates in the endoplasmic reticulum (ER)-Golgi trafficking. This examination identified many novel LetAS-RsmYZ-CsrA regulated effectors which are involved in this process. To further characterize the role of these 26 effectors in vesicular trafficking, they were examined in yeast *arf* and *arl* deletion mutants, which encode small GTPases that regulate ER-Golgi trafficking. This analysis revealed that the effectors examined manipulate different processes of the secretory pathway. Collectively, our results demonstrate that several of the *L. pneumophila* effectors which are coregulated in the bacterial cell are involved in the modulation of the same eukaryotic pathway.**

*Legionella pneumophila* is an opportunistic human pathogen that multiplies within alveolar macrophages and causes a severe pneumonia known as Legionnaires' disease. Human disease occurs when aerosolized *L. pneumophila* is inhaled from man-made or natural freshwater reservoirs harboring the bacteria (1–3). In the environment, *L. pneumophila* multiplies in many different protozoan cells that serve as their training ground for pathogenesis (4–6). In order to establish a replicative niche inside eukaryotic cells, *L. pneumophila* modulates host-cell functions by delivering about 300 effector proteins through the Icm/Dot type IVB secretion system (reviewed in references 7, 8, 9, and 10). Most of these effectors have no homologues in the GenBank, but several of them are homologous to eukaryotic proteins or contain eukaryotic protein motifs (11–13).

The effectors that participate in high numbers in the establishment of the *Legionella*-containing vacuole (LCV) are expected to be regulated at the level of gene expression in order to coordinate a successful infection. To date, three regulatory systems have been shown to directly regulate the expression of effector-encoding genes: (i) the PmrAB two-component system was shown to directly activate the expression of about 40 effector-encoding genes (14, 15); (ii) the CpxRA two-component system was shown to directly activate or repress the expression of several effector-encoding genes as well as *icm*/*dot* genes (16, 17); and (iii) the LetAS-RsmYZ-CsrA regulatory cascade, which includes the LetAS two-component system, the two small RNAs (sRNAs) RsmY and RsmZ, and the posttranscriptional repressor CsrA, was shown to posttranscriptionally repress the translation of three effector-encoding genes (18–20). Furthermore, these three regulatory systems were shown to be part of a regulatory network that regulates the expression of effector-encoding genes (21). Beside these three regulatory systems, other regulators such as RpoS, LqsR, and ArgR were suggested to be involved in the regulation of effector-encoding genes (22–24), however, none of them was shown to directly regulate the expression of these genes.

Components similar to the ones identified in the *L. pneumophila* LetAS-RsmYZ-CsrA regulatory cascade were described before in many Gram-negative bacteria, and these components function similarly in all of them (25–27). In *L. pneumophila*, this regulatory cascade was found to function as follows: during the exponential phase, the CsrA repressor binds to the mRNA of its target genes and represses their translation. Upon entry into the stationary phase, the sensor kinase LetS is activated and phosphorylates LetA, its cognate response regulator. LetA thus binds and activates the expression of the two sRNAs RsmY and RsmZ; these sRNAs contain several CsrA binding sites (AGGA, ATGGA, AC GGA, and AGGGA) and, when expressed, sequester multiple CsrA molecules from their target mRNAs, thus releasing the CsrA repression from its target mRNAs. This leads to high levels of expression of the corresponding proteins at the stationary phase (18–20, 28–30). Examination of mutants lacking different components of this regulatory cascade indicated that LetA is required for intracellular multiplication in amoeba, and the same result was obtained with a double-deletion mutant in the two genes encod-

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ing RsmY and RsmZ (19, 31, 32). The gene encoding CsrA was found to be essential for *L. pneumophila*; however, mutants containing a reduced level of this regulator were shown to be attenuated for intracellular multiplication in amoeba (29, 33).

The regulation of subsets of effectors by the three regulatory systems described above most likely results in groups of effectors which are coordinated at the level of gene expression and might also function together in the host cell. The LCV establishment was shown to be dependent on numerous effectors (8, 34, 35); these effectors modulate many host cell factors involved in vesicular trafficking such as Rab1, Arf1, Sec22b, Sar1, and others (36–39). In addition, effectors involved in the modulation of these factors (such as RalF, SidM/DrrA, and many others) were shown to translocate into host cells very early during infection (39, 40). The members of the subset of effectors regulated by the LetAS-RsmYZ-CsrA cascade are expected to be expressed at the end of an infection cycle (the equivalent of the stationary phase) and probably are translocated into host cells and perform their function early during the next infection, when the LCV is being established.

Currently, the group of effectors regulated by the LetAS-RsmYZ-CsrA cascade consists of three effectors (VipA, LegC7/YlfA, and LegC2/YlfB), all of which were shown to be involved in vesicular trafficking. The VipA effector was identified in a screen looking for proteins that subvert trafficking in yeast, and it was later shown to bind actin *in vitro* and to directly polymerize actin microfilaments. During macrophage infection, VipA was found to be associated with actin patches and early endosomes, suggesting a role in modulating organelle trafficking (41, 42). The paralogous effectors LegC7/YlfA and LegC2/YlfB were identified in a screen looking for proteins that caused a lethal effect on yeast growth, and they were later shown to be involved in vesicular trafficking and to be located within large structures that colocalized with anti-KDEL antibodies in mammalian cells (12, 43). The fact that all the effectors known to be regulated by the LetAS-RsmYZ-CsrA cascade were found to be involved in vesicular trafficking might indicate that additional effectors regulated by this cascade are involved in the same process.

The goal of this research was to identify the group of effectors which are regulated by the LetAS-RsmYZ-CsrA cascade and to find if coregulated effectors also modulate similar eukaryotic pathways. To achieve this goal, we performed a bioinformatic genomic screen with the aim of identifying genes containing putative CsrA regulatory elements. Examination of the genes that were found in the screen resulted in the identification of 26 effectors regulated by the LetAS-RsmYZ-CsrA regulatory cascade. Further work revealed that most of them are involved in modulating the evolutionarily conserved secretory pathway.

## MATERIALS AND METHODS

**Bacterial and yeast strains, plasmids, and primers.** The *L. pneumophila* wild-type strain used in this work was JR32, a streptomycin-resistant, restriction-negative mutant of *L. pneumophila* Philadelphia-1, which is a wild-type strain in terms of intracellular growth (44). In addition, mutant strains derived from JR32 which contain a kanamycin (Km) cassette instead of the *icmT* gene (GS3011) (45), the *letA* gene (OG2001) (31), and the *rsmY* gene (MR-rsmY) (19) and a double-deletion mutant containing a Km cassette instead of the *rsmY* gene and a gentamicin cassette instead of the *rsmZ* gene (MR-rsmYZ) (19) were used. The *Saccharomyces cerevisiae* wild-type strain used in this work was BY4741 (*MAT***a** *his3*Δ *leu2*Δ *met15*Δ *ura3*Δ) (46). In addition, deletion mutants derived from BY4741 (a kind gift from Martin Kupiec, Tel Aviv University) which contain a G418 resistance cassette instead of the *arf1*, *arf2*, *arl1*, *arl3*, and *sec22* genes (47) were used. The plasmids and primers used in this work are listed in Table S2 and Table S3 in the supplemental material.

**Construction of *lacZ* translational fusions.** To generate *lacZ* translational fusions, the regulatory regions of the 62 genes examined were amplified by PCR using the primers listed in Table S3 in the supplemental material. Genes for which the predicted CsrA site was found to be located upstream from the ATG start codon, the translational fusions contain the first seven codons of the gene fused to the *lacZ* gene. When the predicted CsrA site was found to be located downstream from the first ATG, the predicted CsrA site was included in the fusion as well as seven or eight nucleotides downstream of it in such a way that an in-frame *lacZ* fusion was formed. The resulting PCR products were digested with BamHI and EcoRI (or with only EcoRI if a BamHI site was present in the regulatory region), cloned into pGS-lac-02, and sequenced. The list of *lacZ* fusion plasmids constructed is presented in Table S2 in the supplemental material. The β-galactosidase assays were performed as described elsewhere (48).

**Construction of plasmids containing an IPTG-inducible LetA and CsrA.** The plasmids containing the *L. pneumophila letA* and *csrA* genes under the control of P*tac* (pMR-Ptac-csrA-207 and pMR-Ptac-letA-207, respectively) were described before (19). These plasmids were digested with XbaI and EheI, and the fragments containing P*tac-csrA* together with the *lacI* gene, or P*tac-letA* together with the *lacI* gene, were cloned into the plasmids containing the *lacZ* translational fusions of the *mavT*, *mavQ*, and lpg2461 genes, resulting in the plasmids listed in Table S2 in the supplemental material. These plasmids were introduced to different *L. pneumophila* strains and examined using different concentrations of IPTG (isopropyl-β-D-thiogalactopyranoside) (see Results).

**Site-directed mutagenesis of predicted CsrA regulatory elements.** To generate substitutions in the putative CsrA regulatory elements in the regulatory regions of the *cegC1*, *legA7*, *ravH*, *mavT*, *ravR*, *lem11*, *legL3*, *mavQ*, lpg0375, lpg0963, lpg1273, *cegL2*, and lpg2461 genes, site-directed mutagenesis was performed by the PCR overlap extension approach (49) using a method similar to one previously described (15). In genes where the mutations were constructed in putative CsrA sites located upstream from the ATG start codon, the pair of G nucleotides of the CsrA consensus was changed to a pair of C nucleotides; in genes where the putative CsrA site mutated was located downstream from the ATG start codon, the mutations were constructed such that no amino acid was changed (synonymous mutations). The primers used for mutagenesis are listed in Table S3 in the supplemental material, and the plasmids resulting from the site-directed mutagenesis are listed in Table S2 in the supplemental material.

**Construction and examination of *cyaA* fusions and plasmids for expression in yeast.** The pMMB-cyaA-C vector, described before (15), was used to construct CyaA fusions. In addition, a yeast expression vector was constructed to contain the pUC-18 polylinker, at the same reading frame as that present in pMMB-cyaA-C. pUC-18 containing the Km cassette inside its polylinker was digested with EcoRI and PvuII and cloned into pGREG523 (50), and the product was digested with EcoRI and HincII to generate pGREG523-Km. This vector was used to construct effector C-terminal fusions to the 13× *myc* tag regulated by the yeast GAL1 promoter.

The *L. pneumophila* genes examined for translocation and/or lethal effect on yeast growth were amplified by PCR using a pair of primers containing suitable restriction sites (see Table S3 in the supplemental material). The PCR products were subsequently digested with the relevant enzymes and cloned into pUC-18. The insertions of the resulting plasmids were sequenced to verify that no mutations were introduced during the PCR. The insertions were then digested with the same enzymes and cloned into the CyaA and/or the yeast expression vectors; the plasmids generated are listed in Table S2 in the supplemental material. The translocation assays and yeast lethality assays were performed as described before (51).

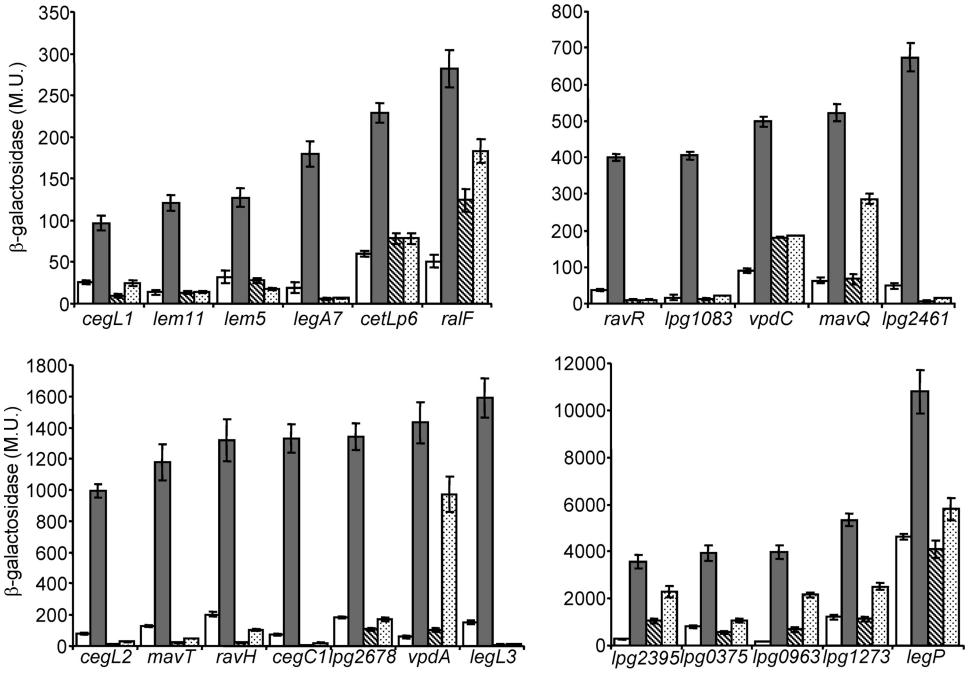


**FIG 1** Numerous *L. pneumophila* effector-encoding genes are activated at the stationary phase in a LetA-RsmYZ-CsrA-dependent manner. The expression of effector translational *lacZ* fusions (the effectors examined are indicated below the bars) was examined in the wild-type strain (JR32) at the exponential phase (white bars) and at the stationary phase (gray bars), in the *letA* deletion mutant (OG2001) at the stationary phase (diagonal striped bars), and in the *rsmYZ* double-deletion mutant (MR-rsmYZ) at the stationary phase (dotted bars). β-Galactosidase activity was measured as described in Materials and Methods. Data (expressed in Miller units [M.U.]) are the averages ± standard deviations (error bars) of the results of at least three different experiments. The effector-encoding genes were divided according to their levels of expression.

## RESULTS

**Numerous *L. pneumophila* genes harbor putative CsrA regulatory elements.** Information available from studies of many bacterial species as well as *L. pneumophila* (19, 25–27) indicated that the CsrA regulatory element consists of the sequence AGGA or ABGGA (B = C, T, or G) and that these sites are usually located in close proximity to the ATG start codon of the regulated genes, as part of their mRNA (52). Most of the genes known to be repressed by CsrA contain at least two CsrA sites, one of which usually overlaps the ribosomal binding site (26, 53). To find *L. pneumophila* genes potentially regulated by CsrA, a genomic search was performed aimed at identifying regulatory regions that contain the following features: (i) at least two putative CsrA sites are present; (ii) at least two of the putative CsrA sites are located less than 50 nucleotides apart; and (iii) one of the putative CsrA sites also constitutes the putative ribosomal binding site of the corresponding gene. This screen resulted in the identification of numerous genes potentially regulated by CsrA. We focused our study on 62 of them (see Table S1 in the supplemental material) grouped according to the following criteria: (i) genes encoding effector proteins (47 genes); (ii) genes encoding regulators (11 genes); and (iii) genes encoding proteins involved in flagellum biosynthesis (4 genes).

It was previously shown that three *L. pneumophila* effector-encoding genes (*legC7-ylfA*, *legC2-ylfB*, and *vipA*) are regulated by the LetA-RsmYZ-CsrA cascade (19). When the level of expression of these genes was examined in a *letA* deletion mutant, a strong reduction in their level of expression was obtained at the stationary phase (19). This reduction occurs because the expression of the RsmY and RsmZ sRNAs was not activated in the *letA* deletion mutant and consequently CsrA continued to repress the mRNA of its target genes also during the stationary phase (19). To examine the genes identified in the bioinformatic screen described above, we constructed translational *lacZ* fusions for all the 62 genes identified and their level of expression was examined in the *L. pneumophila* wild-type and *letA* deletion mutant strains at the stationary phase, as described below.

**Numerous *L. pneumophila* effector-encoding genes are regulated by the LetA-RsmYZ-CsrA regulatory cascade.** The 47 effector-encoding genes identified in the screen included the 3 effector-encoding genes (*legC7-ylfA*, *legC2-ylfB*, and *vipA*) that were shown before to be regulated by the LetA-RsmYZ-CsrA cascade (19) and those encoding 42 known effectors as well as two open reading frames (ORFs) that were not shown before to encode effectors, and their translocation into host cells was validated in this study (see Fig. S1 in the supplemental material). These two novel effectors (lpg1925 and lpg2324) were designated CegL1 and CegL2, respectively, for coregulated with effector genes by the LetA-RsmYZ-CsrA cascade.

Examination of translational *lacZ* fusions constructed for the 44 effectors (see Table S1 in the supplemental material) described above (42 known effectors and the 2 newly identified effectors) indicated that 23 of them had a reduced (between 2- and 242-fold) level of expression in the *letA* deletion mutant in comparison to the wild-type strain at the stationary phase (Fig. 1). To further validate the regulation of these effectors by the LetA-RsmYZ-CsrA regulatory cascade, their level of expression was also examined in the *rsmYZ* double-deletion mutant, and a reduction in their level

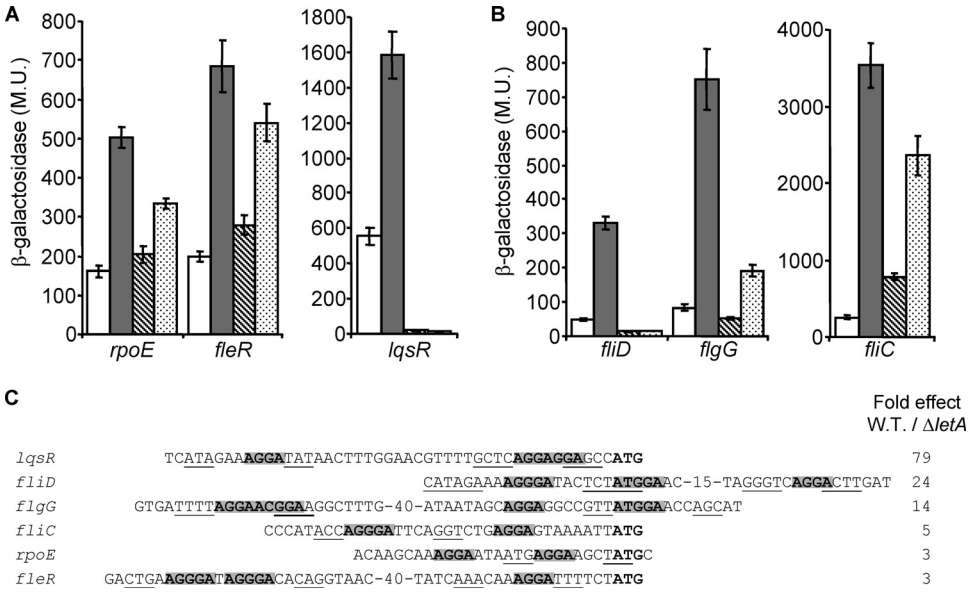


**FIG 2** *L. pneumophila* genes encoding regulators and flagellum biosynthesis proteins are activated at the stationary phase in a LetA-RsmYZ-CsrA-dependent manner. The expression of translational *lacZ* fusions of genes encoding regulators (A) and genes encoding flagellum-related proteins (B) was examined in the wild-type strain (JR32) at the exponential phase (white bars) and at the stationary phase (gray bars), in the *letA* deletion mutant (OG2001) at the stationary phase (diagonal striped bars), and in the *rsmYZ* double-deletion mutant (MR-rsmYZ) at the stationary phase (dotted bars). β-Galactosidase activity was measured as described in Materials and Methods. Data (expressed in Miller units [M.U.]) are the averages ± standard deviations (error bars) of the results of at least three different experiments. The genes were divided according to their levels of expression. (C) The regulatory regions of regulators and flagellum-related genes that were found to be regulated by the LetA-RsmYZ-CsrA regulatory cascade. The nucleotides representing the putative CsrA consensus are in boldface and gray background, small inverted repeats surrounding the putative CsrA sites are underlined, and the ATG start codon is in bold. The genes are indicated on the left, and the fold reduction in the level of expression of each gene between the *letA* deletion mutant and the wild-type (W.T.) strain at the stationary phase is indicated on the right.

of expression was obtained in this mutant as well (Fig. 1). In addition, as expected from genes regulated by the LetA-RsmYZ-CsrA cascade (see the introduction), all these genes were found to have a higher level of expression at the stationary phase than at the exponential phase (Fig. 1). These results indicate that 26 *L. pneumophila* effectors are regulated by the LetA-RsmYZ-CsrA regulatory cascade, which directs their higher level of expression at the stationary phase.

**Regulators and flagellum-related genes are also regulated by the LetA-RsmYZ-CsrA cascade.** Two additional groups of genes were examined for regulation by the LetA-RsmYZ-CsrA cascade: genes encoding regulators and genes encoding proteins involved in flagellum biosynthesis. The motivation to examine regulators of gene expression comes from the observation that the known regulators of *L. pneumophila* effectors form a regulatory network (see the introduction). Therefore, it was interesting to examine whether the LetA-RsmYZ-CsrA cascade controls the expression of regulators that might in turn regulate the expression of other effector-encoding genes, thus expanding the regulatory network of the effectors. Moreover, it was previously shown in several bacterial species that CsrA usually functions as a regulator of regulators (26, 54). The incentive for examining genes that encode proteins involved in flagellum biosynthesis comes from the correlation between the effector and flagellum gene expression (55, 56) and from the fact that the LetA response regulator was first identified in a screen examining differential expression of flagella (18). Using analyses similar to the ones described above, 11 regulators and four flagellum-related genes were examined (see Table S1 in the supplemental material). Three genes encoding regulators (*rpoE*, *lqsR*, and *fleR*) and three genes encoding proteins involved in flagellum biosynthesis (*fliC*, *fliD*, and *flgG*) were found to be regulated by the LetA-RsmYZ-CsrA cascade (Fig. 2A and B, respectively). It should be noted that FleR and LqsR were previously suggested to be involved in flagellum gene expression (57, 58) and that LqsR was also suggested to be involved in the regulation of effector-encoding genes (58). These results show that, in addition to directly contributing to the regulation of effector-encoding genes, the LetA-RsmYZ-CsrA cascade also controls the expression of regulators that by themselves might regulate the expression of other effector-encoding genes.

**Properties of the *L. pneumophila* CsrA regulatory element.** The analyses described above resulted in the identification of 32 genes regulated by the LetA-RsmYZ-CsrA cascade (26 effector-encoding genes, 3 genes encoding regulators, and 3 flagellum-related genes). The majority (18) of these genes contain two putative CsrA sites, and the rest contain 3 to 6 CsrA sites (Fig. 2C and 3; see also Table S1 in the supplemental material). In total, these 32 genes harbor 86 putative CsrA regulatory elements, 53 of which contain an adenosine nucleotide directly upstream from the CsrA consensus sequence (AGGA, ATGGA, ACGGA, or AGGGA). This observation might suggest that adenosine is the preferred nucleotide at this position. Another interesting observation was that the CsrA regulatory element ACGGA was rarely found in the genes identified. Of the 86 potential CsrA sites detected, only four sites, in four different genes (*cegC1*, *cetLp6*, cegL2, and *flgG*), were found to harbor this sequence. In addition, these four genes contain two or three additional CsrA sites, which suggests that, even in these genes, the ACGGA site might not be functional. In correlation


**FIG 3** The putative CsrA regulatory elements of effector-encoding genes. The regulatory regions of the effectors found to be regulated by the LetA-RsmYZ-CsrA cascade are presented. The nucleotides representing the putative CsrA consensus are in boldface and gray background, small inverted repeats surrounding the putative CsrA sites are underlined, the ATG start codon is in bold, and the nucleotides that were mutated are marked with asterisks. The effector designations are indicated on the left, and the fold reduction in the level of expression of each effector between the *letA* deletion mutant and the wild-type strain at the stationary phase is indicated on the right.

with this finding, when we examined the *L. pneumophila* RsmY and RsmZ sRNAs, it was found that, of the 11 potential CsrA sites present in these two sRNAs, only one potential site consists of the sequence ACGGA (data not shown). On the other hand, the most abundant CsrA site was found to be AGGA; this site appeared in 50 of the 86 potential CsrA sites detected, and 32 of these sites are composed of the sequence AAGGA. These results further refine our understanding of the CsrA consensus used by the *L. pneumophila* CsrA posttranscriptional repressor and indicate that there is a preference for the use of an AGGA site whereas the ACGGA site is rarely used.

**Different components of the LetA-RsmYZ-CsrA regulatory cascade similarly affect the expression of effectors.** Analysis of the expression data of the genes regulated by the LetA-RsmYZ-CsrA regulatory cascade indicates that the degrees of reduction in their levels of expression in the *letA* deletion mutant and the *rsmYZ* double-deletion mutant differ significantly from the those seen with the wild-type strain (Fig. 1, 2, and 3). To further substantiate the regulation of these genes by the LetA-RsmYZ-CsrA regulatory cascade, we examined the effect of overexpression of different components of this cascade on the levels of expression of three effector-encoding genes (lpg2461, *mavT*, and *mavQ*), which were affected differently by the *letA* deletion mutant (129-fold, 50-fold, and 8-fold reduction, respectively). The levels of expression of these three genes were examined under conditions of increasing levels of CsrA (using an IPTG [isopropyl-β-D-thiogalactopyranoside]-inducible P*tac*-CsrA construct) in the wild-type strain and in the *rsmY* deletion mutant (Fig. 4A). Increasing levels of CsrA in the wild-type strain reduced the level of expression of lpg2461 in the two higher concentrations of IPTG (0.1 mM and 1 mM). The reduction occurring with *mavT* was observed only with the maximal IPTG concentration, and no reduction in the level of expression of *mavQ* was observed in the wild-type strain (Fig. 4A). When the same analysis was performed in the *rsmY* deletion mutant, the levels of expression of all three genes were reduced. A reduction in the level of expression of lpg2461 was obtained even when using a very low concentration of IPTG (0.01 mM). Furthermore, the levels of expression of these genes were also examined under conditions of increasing levels of LetA (using a P*tac*-LetA construct) in the *letA* deletion mutant and in the *rsmYZ* double-deletion mutant (Fig. 4B). When increasing levels of LetA were examined in the *letA* deletion mutant (Fig. 4B), they were seen to result in complementation of the levels of expression of the three *lacZ* fusions. This complementation was completely dependent on the presence of RsmY and RsmZ, since, in their absence (the *rsmYZ* double-deletion mutant), no increases in the levels of ex-

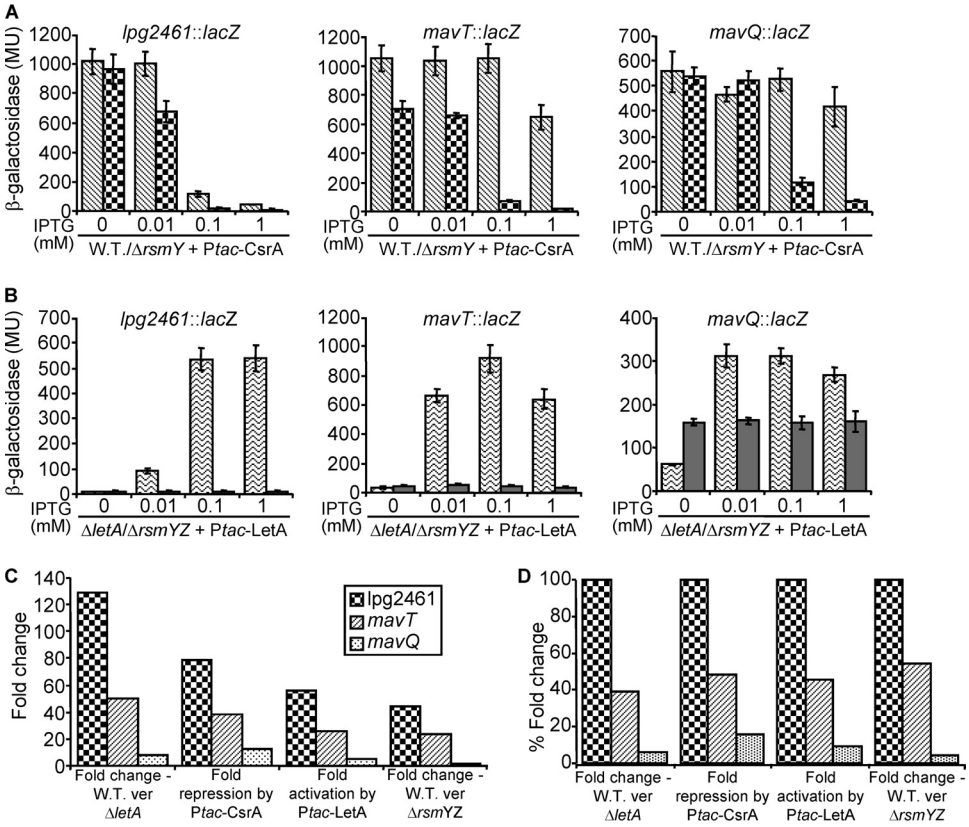


**FIG 4** CsrA and LetA affect the levels of expression of different effector *lacZ* fusions similarly. (A) The levels of expression of the lpg2461, *mavT*, and *mavQ lacZ* fusions were examined in *L. pneumophila* wild-type strain JR32 (diagonal striped bars) and *L. pneumophila rsmY* deletion mutant MR-rsmY (boxed bars). The bacteria examined contained a plasmid with the *csrA* gene cloned under the control of the P*tac* promoter (activated by IPTG). (B) The same effector *lacZ* fusions were examined in the *L. pneumophila letA* deletion mutant OG2001 (waved bars) and the *L. pneumophila rsmYZ* double-deletion mutant MR-rsmYZ (gray bars). The bacteria examined contained a plasmid with the *letA* gene cloned under the control of the P*tac* promoter. In the experiments represented in both panel A and panel B, the strains were grown in media containing different concentrations of IPTG (indicated below the bars) and β-galactosidase activity was measured at the stationary phase as described in Materials and Methods. The data are the averages ± standard deviations (error bars) of the results of at least three different experiments. (C) Comparison of the effects of different mutants and overexpression conditions on the level of expression of the effector *lacZ* fusions examined as described for panels A and B. The mutants and overexpression conditions compared are indicated below the bars. (D) Data represent the results of experiments performed as described for panel C, but the degree of the effect on lpg2461 was normalized to 100% and the relative effects on *mavT* and *mavQ* genes were calculated in relation to it. ver, versus.

pression of the three effector *lacZ* fusions were obtained with increasing levels of LetA (Fig. 4B). In this analysis, the maximal activation by LetA was obtained with lpg2461 and the lowest with MavQ.

In all parameters tested in Fig. 1 and Fig. 4, the strongest effect was obtained with lpg2461, a moderate effect was observed with *mavT*, and the weakest effect was seen with *mavQ* (Fig. 4C). After normalization according to the effect on lpg2461, the degrees of the effects of each of the mutants or overexpression conditions on the genes were similar (Fig. 4D). These results strongly indicate that, even though the degrees of the effects of the *letA* deletion mutant on the levels of expression of the genes examined were different, all of them are regulated by the LetA-RsmYZ-CsrA regulatory cascade.

**Mutagenesis of the putative CsrA regulatory elements results in elevated levels of expression at the exponential phase.** To further investigate the putative CsrA sites identified in the bioinformatic screen, we performed site-directed mutagenesis on 13 putative CsrA sites in 13 different effector-encoding genes (marked by asterisks in Fig. 3). The putative CsrA sites selected for site-directed mutagenesis were from genes that were affected differently by the *letA* deletion mutant, and they were constructed in putative CsrA sites located upstream or downstream from the ATG start codon of the genes (Fig. 3). In addition, we avoided constructing mutations in putative CsrA sites that also constitute the potential ribosomal binding sites, since generating mutations in such sites would most likely influence both the putative CsrA site and the putative ribosomal binding site, making the results ambiguous. The results of this analysis were very clear (Fig. 5): mutations in all the putative CsrA sites (9 AGGA, 4 ATGGA, and 3 AGGGA sites) resulted in levels of expression of the *lacZ* fusions that were higher than those seen with the wild-type *lacZ* fusions at the exponential phase. This result was expected, since these sites are subjected to repression by the CsrA translational repressor at the exponential phase (see the introduction). In addition, we mutagenized the ACGGA site found in *cegC1*, and the level of expression of a *lacZ* fusion containing this mutation was found to be similar to that of the wild-type *lacZ* fusion (data not shown). This result supports our assumption that the ACGGA sites are not functional CsrA sites in *L. pneumophila* (see above). Collectively,

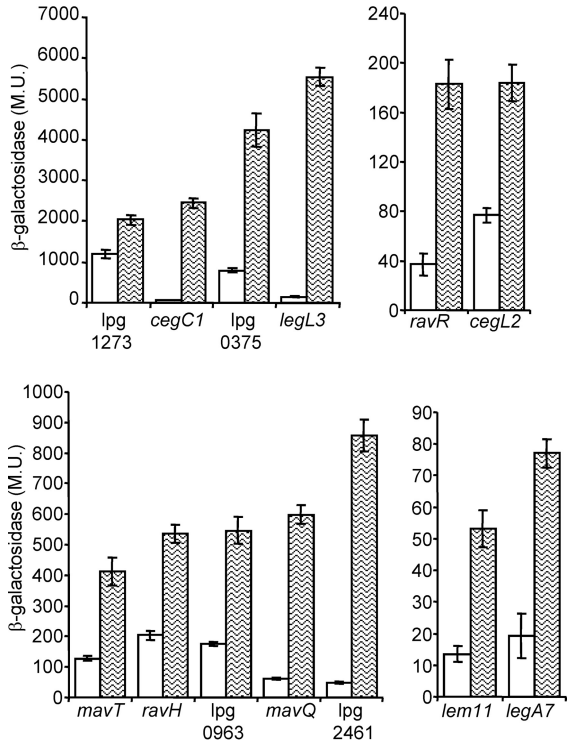


**FIG 5** Mutations constructed in the putative CsrA regulatory elements resulted in elevated levels of expression at the exponential phase. The expression of effector (indicated below the bars) wild-type *lacZ* fusions (white bars) and *lacZ* fusions of the same genes containing a mutation in a putative CsrA binding site (waved bars) were examined at the exponential phase in the *L. pneumophila* wild-type strain. The mutations constructed are marked by asterisks in Fig. 3. β-Galactosidase activity was measured as described in Materials and Methods. The data are the averages ± standard deviations (error bars) of the results of at least three different experiments. The genes were divided according to their levels of expression.

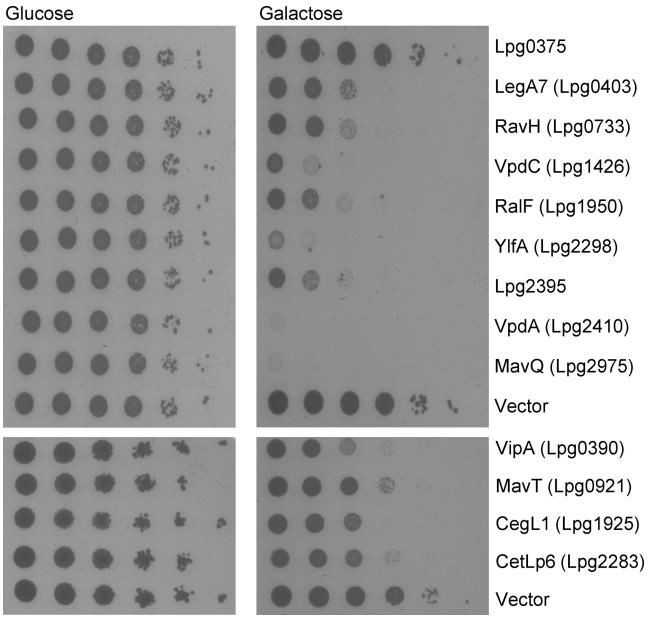


**FIG 6** Effectors regulated by the LetA-RsmYZ-CsrA cascade cause different degrees of lethal effect on yeast growth. The effectors regulated by the LetA-RsmYZ-CsrA cascade were cloned under the control of the GAL1 promoter and grown on plates containing glucose or galactose (inducing conditions) in the wild-type *S. cerevisiae* BY4741 strain. Ten-fold serial dilutions were performed, and the lethal effect was compared to the one of the vector pGREG523 control (vector). Lpg0375 is presented as a representative of an effector that caused no lethal effect on yeast growth. The effectors presented in the upper panel were examined at 30°C, and the effectors presented in the lower panel were examined at 37°C.

these results strongly indicate that the mutated CsrA sites are subjected to repression at the exponential phase and that their mutagenesis resulted in a relief of this repression, thus further supporting the idea of the regulation of these genes by the LetA-RsmYZ-CsrA cascade.

**The LetA-RsmYZ-CsrA coregulated effectors inhibit yeast cell growth.** Five of the 26 effector proteins identified in our screen (VipA, VpdC, RalF, LegC7/YlfA, and VpdA) were shown before to be lethal when overexpressed in yeast cells (43, 59, 60). The lethal effect on yeast growth suggests that a conserved and essential eukaryotic process which is modulated by the effector in the host cell was also modulated in the yeast cell, resulting in an inhibition of cell growth (43, 59, 61, 62). Examination of all the 26 LetA-RsmYZ-CsrA coregulated effectors in yeast revealed that 12 of them (VipA, LegA7, RavH, MavT, VpdC, CegL1, RalF, CetLp6, LegC7/YlfA, Lpg2395, VpdA, and MavQ) cause lethal effects on yeast growth (Fig. 6 and Table 1). (Lpg2461 was not examined since it was impossible to introduce it into yeast; a similar result was observed before with other effectors, some of which were found to inhibit translation [59].) This result indicates that almost half of the LetA-RsmYZ-CsrA coregulated effectors affect conserved eukaryotic processes.

**Most of the LetA-RsmYZ-CsrA coregulated effectors manipulate vesicular trafficking in yeast.** To further examine these effectors, we utilized a well-established approach which was used before to uncover genetic interactions, such as synthetic growth defects, between gene pairs in yeast (64). This approach was also used before with bacterial effectors, some of which resulted in a synthetic growth defect when expressed in specific yeast deletion mutants related to their function (i.e., the mutants were hypersensitive to the expression of the effector) (51, 65). The assumption is that this phenotype results from the activity of the effector, which resembles the phenotype of a mutation in the effector target protein; thus, the phenotype results from the combined effect of the absence of the gene deleted and the misfunctioning of the effector target protein. Three of the LetA-RsmYZ-CsrA coregulated effectors (VipA, RalF, and LegC7/YlfA) which were shown to cause lethal effect on yeast growth and one that did not (LegC2/YlfB) were shown before to be involved in vesicular trafficking (42, 59, 66). Therefore, we decided to test whether additional LetA-RsmYZ-CsrA coregulated effectors are involved in vesicular trafficking.

Most of the genes encoding components of the secretory pathway are essential for yeast growth. Exceptions to this rule are genes such as *sec22* and *arf1*, which can be deleted (67, 68). Therefore, we overexpressed the LetA-RsmYZ-CsrA coregulated effectors in yeast deleted for the *sec22* gene, which encodes an R-SNARE protein (involved in ER-Golgi trafficking), and examined their effect on the growth of this mutant. As expected from their known function, VipA, RalF, and LegC2/YlfB showed an enhanced lethal effect on yeast growth in the *sec22*Δ mutant in comparison to their effect on the wild-type yeast strain (Fig. 7 and data not shown).

**TABLE 1** Effect on yeast growth and trafficking caused by LetA-RsmYZ-CsrA-regulated effectors

| lpg no. | Name | Lethal effect in[a]: Wild type | Lethal effect in[a]: *sec22*Δ mutant | Involvement in trafficking[b] | Source or reference(s)[c] |
|---|---|---|---|---|---|
| lpg0012 | CegC1 | − | +++ | | |
| lpg0375 | | − | − | SGD in *arl1*Δ mutant | This study |
| lpg0390 | VipA | + (37°C) | ++++ (37°C) | Binds actin, associates with early endosomes | 42, 59 |
| lpg0403 | LegA7 | ++ | ++++ | | |
| lpg0733 | RavH | ++ | − | SGD in *arf*Δ and *arl*Δ mutants | This study |
| lpg0921 | MavT | + (37°C) | ++ (37°C) | | |
| lpg0963 | | − | ++ | | |
| lpg1083 | | − | ++ | | |
| lpg1110 | Lem5 | − | ++ | | |
| lpg1166 | RavR | − | ++ | | |
| lpg1273 | | − | ++ | | |
| lpg1426 | VpdC | +++ | ++++ | Phospholipase A | 59 |
| lpg1598 | Lem11 | − | ++ | | |
| lpg1660 | LegL3 | − | ++ | | |
| lpg1884 | LegC2/YlfB | − | ++ | Colocalized with anti-KDEL antibodies | 43, 59 |
| lpg1925 | CegL1 | + (37°C) | +++ (37°C) | | |
| lpg1950 | RalF | ++ | +++ | Arf1-GEF | 43, 59, 63 |
| lpg2283 | CetLp6 | + (37°C) | +++ (37°C) | SGD in *arf*Δ and *arl*Δ mutants | This study |
| lpg2298 | LegC7/YlfA | ++++ | ++++ | Colocalized with anti-KDEL antibodies | 12, 43, 59 |
| lpg2324 | CegL2 | − | +++ | | |
| lpg2395 | | ++ | +++ | | |
| lpg2410 | VpdA | ++++ | ++++ | Phospholipase A | 59 |
| lpg2461 | | X | X | | |
| lpg2678 | | − | − | | |
| lpg2975 | MavQ | ++++ | ++++ | | |
| lpg2999 | LegP | − | − | | |

[a] The scale of the lethal effect on yeast growth was as follows: −, no effect; +, weak effect; ++, medium effect; +++, strong effect; ++++, very strong effect; X, no yeast transformants were obtained.
[b] SGD, synthetic growth defect.
[c] The references listed indicate the sources for the information about the effect on trafficking.

Furthermore, 15 additional effectors showed a clear synthetic growth defect in the *sec22*Δ mutant in comparison to the wild-type yeast (Fig. 7 and Table 1). Only 6 of the 25 effectors examined did not show synthetic growth defect in the *sec22*Δ mutant (Fig. 7 and Table 1). These six effectors include three effectors that caused a very strong lethal effect on wild-type yeast growth (no additive effect was observed for two of the three [Fig. 6]) and three effectors that did not cause a lethal effect on wild-type yeast growth. The lack of an additive effect with these effectors further supports the idea of the specificity of the synthetic growth defect observed with the majority of the effectors. Strikingly, the lethal effect mediated by RavH in the wild-type yeast was completely suppressed in the

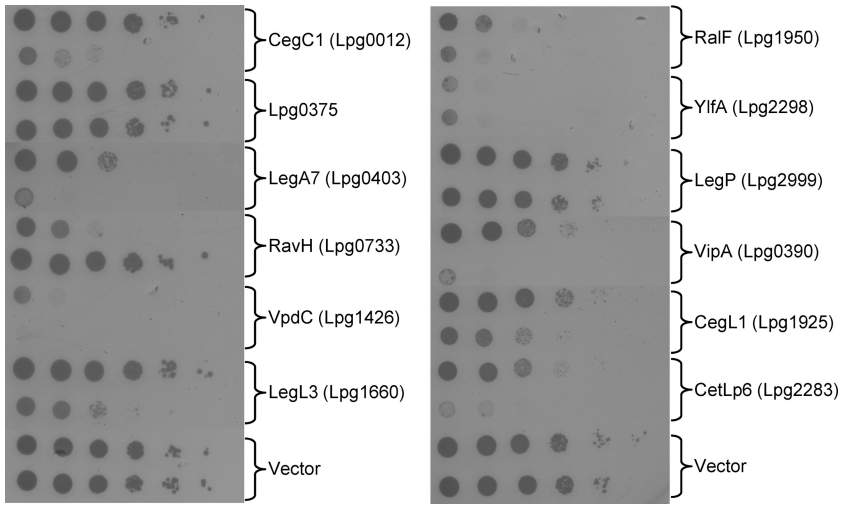


**FIG 7** Several *L. pneumophila* effectors cause a synthetic growth defect in the yeast *sec22* deletion mutant. Data represent a comparison of the degrees of lethal effect caused by effectors expressed in the wild-type yeast and the *sec22*Δ mutant. Yeast containing the effectors indicated on the right were plated in 10-fold serial dilutions under inducing conditions (galactose). The effectors were overexpressed in the wild-type *S. cerevisiae* BY4741 strain (upper dilutions in each pair) and the *sec22*Δ mutant (lower dilutions in each pair). The vector on which the effectors were cloned (pGREG523) was used as a control (vector). Lpg0375, YlfA, and LegP are presented as representatives of effectors that caused no additive effect in the *sec22*Δ mutant. The glucose control plates of this experiment are shown in Fig. S2 in the supplemental material.

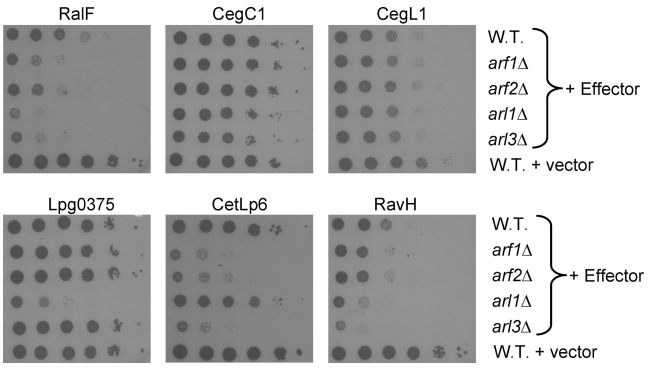


**FIG 8** Several *L. pneumophila* effectors cause a synthetic growth defect in the yeast *arf* and *arl* deletion mutants. Data represent a comparison of the degrees of lethal effect caused by RalF, CegC1, CegL1, lpg0375, CetLp6, and RavH in different yeast *arf* and *arl* deletion mutants plated in 10-fold serial dilutions under inducing conditions (galactose). The effectors (indicated above each panel) were overexpressed in the wild-type *S. cerevisiae* BY4741 strain (W.T.), the *arf1* deletion mutant (*arf1*Δ), the *arf2* deletion mutant (*arf2*Δ), the *arl1* deletion mutant (*arl1*Δ), and the *arl3* deletion mutant (*arl3*Δ). The vector on which the effectors were cloned (pGREG523) was used as a control (vector). CegC1 and CegL1 are presented as representatives of effectors that caused no additive effect in the *arf* and *arl* deletion mutants. The glucose control plates of this experiment are shown in Fig. S4 in the supplemental material.

*sec22*Δ mutant (Fig. 7), indicating that deletion of the *sec22* gene can sometimes prevent the lethal effect caused by an effector (see below). To further strengthen the specificity of the results obtained, we examined two effectors that are not involved in trafficking (LegS2 and LpdA [51, 69]), and that are not regulated by the LetA-RsmYZ-CsrA cascade, for their lethal effect in the *sec22*Δ mutant in comparison to the wild-type yeast strain; no additive effect was observed (see Fig. S3 in the supplemental material). Together, these results strongly indicate that 19 of the 26 LetA-RsmYZ-CsrA coregulated effectors are probably involved in the modulation of endoplasmic reticulum (ER)-Golgi vesicular trafficking in yeast.

**Three novel LetA-RsmYZ-CsrA coregulated effectors manipulate different components of ER-Golgi vesicular trafficking.** It was shown before that effectors manipulate different components of the secretory pathway (34, 35, 70), and the effectors that showed a synthetic growth defect in the *sec22*Δ mutant described above might function on different components of this pathway. To further characterize the involvement of these effectors in the ER-Golgi trafficking, they were expressed in *arf1*Δ, *arl1*Δ, and *arl3*Δ mutants, which encode small GTPases involved in the ER-Golgi trafficking.

The results obtained from this analysis indicated that the expression of four effectors (Lpg0375, RalF, CetLp6, and RavH) caused a synthetic growth defect in the *arf1*Δ, *arl1*Δ, and *arl3*Δ mutants (Fig. 8 and Table 1). This result probably indicates that the *arf1*Δ, *arl1*Δ, and *arl3*Δ mutants uncover effects on the secretory pathway that are more specific than those of the *sec22*Δ mutant. To further strengthen the specificity of the results obtained, we examined two effectors (LegS2 and LpdA) that are not regulated by the LetA-RsmYZ-CsrA cascade for their lethal effect in the *arf1*Δ, *arf2*Δ, *arl1*Δ, and *arl3*Δ mutants in comparison to the wild-type yeast strain, and no additive effect was observed (see Fig. S3A in the supplemental material). In addition, the mutants strains themselves grew similarly to the wild-type yeast strain under the conditions used (see Fig. S3B).

One of the effectors that caused a synthetic growth defect in the *arf* and *arl* mutants was RalF (Fig. 8). RalF was shown before to function as an Arf1-GEF (ADP ribosylation factor-guanine exchange factor) (43, 59, 63), and therefore it was expected that it would cause a synthetic growth defect in these mutants (see Discussion). Three additional effectors examined (Lpg0375, CetLp6, and RavH) showed interesting results in the *arf* and *arl* mutants. (i) The Lpg0375 effector showed no additive effect in the *sec22*Δ mutant as well as in the *arf1*Δ, *arf2*Δ, and *arl3*Δ mutants but had a very strong lethal effect in the *arl1*Δ deletion mutant (Fig. 8). This result might indicate that this effector modulates a specific factor of the secretory pathway and that its malfunctioning caused the synthetic growth defect only in the *arl1*Δ mutant. (ii) The CetLp6 effector caused a moderate lethal effect on wild-type yeast growth, and it showed a strong additive lethal effect in the *sec22*Δ mutant as well as in the *arf1*Δ, *arf2*Δ, and *arl3*Δ mutants, but no additive effect was observed in the *arl1*Δ deletion mutant (Fig. 8). The contrasting results obtained with Lpg0375 and CetLp6 in all the mutants examined might indicate that the *arl1*Δ mutant exposes specific functions mediated by the effectors. (iii) The most fascinating result was obtained with the effector RavH. RavH caused moderate lethal effect on wild-type yeast growth, and its lethal effect was completely suppressed in the *sec22*Δ mutant (Fig. 7). However, expression of this effector in the *arf1*Δ, *arf2*Δ, *arl1*Δ, and *arl3*Δ mutants showed a synthetic growth defect, especially with the *arl3*Δ mutant (Fig. 8), indicating that this effector modulates a host factor different from those modulated by the other effectors examined (see Discussion). Collectively, these results indicate that three novel LetA-RsmYZ-CsrA coregulated effectors probably manipulate different components of the ER-Golgi trafficking pathway.

## DISCUSSION

The study of the regulation of *L. pneumophila* effectors until now revealed three main regulatory systems that control the level of expression of effectors (21). These systems include the two-component systems PmrAB and CpxRA and the LetA-RsmYZ-CsrA regulatory cascade (references 15, 16, and 19 and this study). These three regulatory systems were shown to be part of a regulatory network that regulates the expression of effector-encoding genes (21). As part of this network, a regulatory switch was described in which the PmrA response regulator directly activates the expression of a group of effector-encoding genes, and indirectly represses the expression of a second group of effectors, by directly activating the expression of the CsrA posttranscriptional repressor (19). In this study, we expanded the group of effector-encoding genes known to be regulated by the LetA-RsmYZ-CsrA regulatory cascade and identified three regulators that might also participate in the regulatory network that controls the expression of effector-encoding genes. We found that the CsrA posttranscriptional repressor itself controls the expression of three other regulators (FleR, RpoE, and LqsR). The possible involvement of FleR in the regulation of pathogenesis-related genes is the most appealing one, since its regulation of a flagellum-related gene in *L. pneumophila* was found to be different from its regulation in other bacteria (57), which might indicate that this regulator participates in the regulation of genes other than flagellum-related genes in *L. pneumophila*.

The level of expression of the LetA-RsmYZ-CsrA coregulated effectors was found to be higher at the stationary phase (reference

19 and this study). This result suggests that the cytoplasm of *L. pneumophila* that ended an infection cycle (the equivalent of the stationary phase) should be loaded with the 26 LetA-RsmYZ-CsrA coregulated effectors identified in this study. Thus, when a new infection cycle begins, these effectors are probably the first to translocate into the host cell (depending also on their secretion signal and possible interaction with chaperons) and might participate in the establishment of the LCV which involves massive modulation of the ER-Golgi vesicular trafficking. To examine whether the LetA-RsmYZ-CsrA coregulated effectors are involved in vesicular trafficking, we examined them in several yeast mutants with mutations in genes which encode proteins that participate in this pathway. The Sec22 R-SNARE protein is a critical component of the yeast secretory pathway, and it functions in ER-Golgi anterograde and retrograde trafficking (71). However, a deletion of the yeast *sec22* gene was found to be viable since another R-SNARE protein (Ykt6) compensates for its absence (67). Moreover, it was previously shown that the human Sec22b protein (the homolog of the yeast Sec22 protein) is localized to the LCV during *L. pneumophila* infection (38) and that this protein is required for *L. pneumophila* multiplication in cells (36, 37). Additionally, in yeast there are six (Sar1, Arf1, Arf2, Arl1, Arl3, and Ypt1) small GTPases that directly regulate different processes of the secretory pathway, and effectors that modulate Arf1 and Ypt1 were already described (39, 40, 43, 59, 63). Since *sar1* and *ypt1* are essential genes in yeast, we used yeast *arf1*Δ, *arf2*Δ, *arl1*Δ, and *arl3*Δ mutants in our analyses. Arf1 and Arf2 are highly homologous; they regulate ER-Golgi trafficking and are synthetic lethal in yeast (68), and the Arl1 and Arl3 small GTPases are involved in Golgi trafficking (72).

These analyses uncovered three interesting effectors. The first effector, CetLp6, like RalF, showed a synthetic growth defect in the *sec22*Δ mutant as well as in the *arf1*Δ, *arf2*Δ, and *arl3*Δ mutants (RalF showed a synthetic growth defect also in the *arl1*Δ mutant; Fig. 8). It is known that RalF functions as an Arf1-GEF; therefore, it was expected that a synthetic growth defect would be obtained with this effector in the *arf1*Δ and *arf2*Δ mutants since in both mutants the level of the RalF target protein is reduced and consequently RalF is expected to activate a larger fraction of its remaining target protein, thus causing a synthetic growth defect. The similar result that was obtained with CetLp6 might indicate that this effector functions on one of the Arf/Arl proteins as well. The second effector, Lpg0375, showed a result that was opposite the CetLp6 result, and a synthetic growth defect was observed only in the *arl1*Δ mutant. Since the function of Arl1 is restricted to the Golgi compartment, this result might indicate that this effector modulates a protein of the secretory pathway that functions in this compartment. The third effector, RavH, showed intriguing results that might indicate its function. The moderate lethal effect of RavH in wild-type yeast was completely suppressed in the *sec22*Δ mutant, but its expression caused a synthetic growth defect with the *arf1*Δ, *arf2*Δ, and *arl1*Δ mutants and particularly in the *arl3*Δ mutant. In general, suppression of the lethal effect of an effector can be obtained in one of three ways: (i) deletion of the yeast target protein modulated by the effector (as was shown with the effector LecE and its target protein Pah1 [51]); (ii) overexpression of a yeast protein that counteracts the effector function (as was shown with the effector LecE and the Dgk1 protein [51]); or (iii) overexpression of the effector target protein (as was shown with the effector AnkX and its target protein Ypt1 [73]). The results obtained with RavH in the *sec22*Δ mutant might indicate that Sec22 itself or a protein located upstream in the pathway which leads to Sec22 activation (such as Sar1 or a Sar1-GEF) might serve as the RavH target protein.

In conclusion, our study revealed numerous effectors regulated by the LetA-RsmYZ-CsrA regulatory cascade, most of which were found to be implicated in vesicular trafficking. Further studies that will result in the identification of the host proteins that interact with these coregulated effectors are required in order to determine their specific function.

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