JOURNAL OF BACTERIOLOGY, Jan. 2002, p. 67–75 Vol. 184, No. 1
0021-9193/02/$04.00+0 DOI: 10.1128/JB.184.1.67–75.2002

Characterization of a *Legionella pneumophila relA* Insertion Mutant and Roles of RelA and RpoS in Virulence Gene Expression

Tal Zusman, Ohad Gal-Mor, and Gil Segal*

Department of Molecular Microbiology & Biotechnology, George S. Wise Faculty of Life Sciences, Tel-Aviv University, Ramat-Aviv, Tel-Aviv 69978, Israel

Received 30 July 2001/Accepted 27 September 2001

To investigate the involvement of RelA in the regulation of *Legionella pneumophila* virulence, a deletion substitution was constructed in the *relA* gene. The *relA* knockout resulted in an undetectable level of ppGpp in the cells during the stationary phase, but the original level was restored when the *relA* gene product was supplied on a plasmid. The effect of the *relA* mutation was examined with two systems that are known to be expressed during the stationary phase in *L. pneumophila*. Pigment production was found to be dependent on the *relA* gene product, and only one-half as much pigment was produced by the *relA* mutant as by the wild-type strain. Flagellum gene expression was also found to be dependent on the *relA* gene product, as determined with a *flaA*::*lacZ* fusion. However, the *relA* gene product was found to be dispensable for intracellular growth both in HL-60-derived human macrophages and in the protozoan host *Acanthamoeba castellanii*. To determine the involvement of the *relA* gene product in expression of *L. pneumophila* genes required for intracellular growth (*icm*/*dot* genes), nine *icm*::*lacZ* fusions were constructed, and expression of these fusions in the wild-type strain was compared with their expression in *relA* mutant strains. Expression of only one of the *icm*::*lacZ* fusions was moderately reduced in the *relA* mutant strain. Expression of the nine *icm*::*lacZ* fusions was also examined in a strain containing an insertion in the gene that codes for the stationary-phase sigma factor RpoS, and similar results were obtained. We concluded that RelA is dispensable for intracellular growth of *L. pneumophila* in the two hosts examined and that both RelA and RpoS play minor roles in *L. pneumophila icm*/*dot* gene expression.

Legionella pneumophila, the causative agent of Legionnaires' disease, is a facultatively intracellular pathogen. *L. pneumophila* is able to infect, multiply within, and kill human macrophages, as well as free-living amoebae (27, 34). The bacteria are taken up by regular phagocytosis or by a special mechanism termed coiling phagocytosis (6, 25). The bacteria are then found within a specialized phagosome that does not fuse with lysosomes or acidify (6, 23, 26), and the specialized phagosome undergoes several recruitment events that include association with smooth vesicles, mitochondria, and rough endoplasmic reticulum (1, 24, 46). The bacteria multiply within the specialized phagosome until the cell eventually lyses, releasing bacteria that can start new rounds of infection (27, 34).

Two regions of genes required for human macrophage killing and intracellular multiplication have been discovered in *L. pneumophila* (reviewed in references 39 and 48). Region I contains seven genes (*icmV*, *-W*, and *-X* and *dotA*, *-B*, *-C*, and *-D*) (5, 7, 30, 47), and region II contains 17 genes (*icmT*, *-S*, *-R*, *-Q*, *-P*, *-O*, *-N*, *-M*, *-L*, *-K*, *-E*, *-G*, *-C*, *-D*, *-J*, *-B*, and *-F*) (3, 32, 37, 38, 47). Most of these genes have also been shown to be required for intracellular growth in the protozoan host *Acanthamoeba castellanii* (41). Fourteen of the Icm/Dot proteins (IcmT, -P, -O, -L, -K, -G, -C, -D, -J, and -B and DotA, -B, -C, and -D) have been found to exhibit significant sequence similarity with Tra/Trb proteins from the IncI plasmids colIb-P9 and R64 (28, 42).

* Corresponding author. Mailing address: Department of Molecular Microbiology & Biotechnology, George S. Wise Faculty of Life Sciences, Tel-Aviv University, Ramat-Aviv, Tel-Aviv 69978, Israel. Phone: 972-3-6405287. Fax: 972-3-6409407. E-mail: GilS@tauex.tau.ac.il.

At this time, there is very little information regarding regulation of *L. pneumophila* virulence and no information regarding regulation of *icm*/*dot* gene expression. So far, the stationary-phase sigma factor RpoS (encoded by the *rpoS* gene) has been shown to be involved in *L. pneumophila* virulence, and a strain containing a knockout in this gene lost the ability to grow in the protozoan host *A. castellanii* (17) and was attenuated for intracellular growth in murine bone marrow-derived macrophages (4). However, this gene has been found to be dispensable for growth in HL-60-derived human macrophages and in THP-1 cells (17). It has been suggested that another factor that is related to the stationary phase, the *relA* gene product, is involved in regulation of *L. pneumophila* pathogenicity (18). RelA is a ppGpp synthatase that plays a major role in the *Escherichia coli* stringent response (10). The stringent response is a global response that occurs as a consequence of the binding of uncharged tRNA to ribosomes, which activates the RelA enzyme. As a result of this activation, ppGpp accumulates in the cells, which leads to rapid inhibition of stable RNA synthesis and accumulation of the stationary sigma factor (RpoS) (10, 16). In other bacteria, the *relA* gene product has been shown to coordinate entry into the stationary phase with several systems, such as fruiting body formation in *Myxococcus xanthus* (19) and morphological differentiation and antibiotic production in *Streptomyces coelicolor* (12). It has been suggested that in *L. pneumophila* this gene product coordinates virulence with entry into the stationary phase (18).

We were interested in determining the precise role of the *relA* gene product in *L. pneumophila* pathogenesis and *icm*/*dot* gene expression. To do this, we constructed a deletion substitution in the *relA* gene on the *L. pneumophila* chromosome and tested its effect on *L. pneumophila* intracellular growth and

TABLE 1. Plasmids used in this study

Plasmid	Features	Reference or source
pAB-1	*lacZ* gene under P*tac* control in pMMB207	36
pGS-flaA-01	*L. pneumophila flaA* regulatory region in pUC18	This study
pGS-flaA-02	*L. pneumophila flaA* regulatory region in pMC1403	This study
pGS-flaA-03	*L. pneumophila flaA* regulatory region in pGS-lac-01	This study
pGS-lac-01	pAB-1 with a promoterless *lacZ* gene	This study
pGS-reg-F1	Regulatory region of *icmF* in pUC18	This study
pGS-reg-F2	Regulatory region of *icmF* in pMC1403	This study
pGS-reg-F3	Regulatory region of *icmF* in pGS-lac-01	This study
pGS-reg-M1	Regulatory region of *icmM* in pUC18	This study
pGS-reg-M2	Regulatory region of *icmM* in pMC1403	This study
pGS-reg-M3	Regulatory region of *icmM* in pGS-lac-01	This study
pGS-relA-01	*L. pneumophila relA* gene in pUC-18	This study
pGS-relA-01-Km	pGS-RelA-01 with the Km cassette in the *relA* gene	This study
pGS-relA-01-Km-GR	Insert of pGS-RelA-01-Km in pLAW344	This study
pGS-relA-05	*L. pneumophila relA* in pMMB207αb	This study
pKP-Q-1	Regulatory region of *icmQ* in pUC18	This study
pKP-Q-21	Regulatory region of *icmQ* in pMC1403	This study
pLAW344	*sacB* MCS *oriT*(RK2) Cm^r *oriR*(ColE1) Ap^r	50
pMC1403	Promoterless *lacZ* in pBR322	8
pMMB207αb	RSF1010 derivative, IncQ *lacI*^{q} Cm^r *oriT* MCS	38
pOG-J-103	Regulatory region of *icmJ* in pUC18	This study
pOG-J-109	Regulatory region of *icmJ* in pMC1403	This study
pOG-J-122	Regulatory region of *icmJ* in pGS-lac-01	This study
pOG-P-105	Regulatory region of *icmP* in pUC18	This study
pOG-P-108	Regulatory region of *icmP* in pMC1403	This study
pOG-P-121	Regulatory region of *icmP* in pGS-lac-01	This study
pOG-Q-126	Regulatory region of *icmQ* in pGS-lac-01	This study
pOG-R-125	Regulatory region of *icmR* in pGS-lac-01	This study
pOG-T-104	Regulatory region of *icmT* in pUC18	This study
pOG-T-107	Regulatory region of *icmT* in pMC1403	This study
pOG-T-120	Regulatory region of *icmT* in pGS-lac-01	This study
pOG-V-110	Regulatory region of *icmV* in pMC1403	This study
pOG-V-123	Regulatory region of *icmV* in pGS-lac-01	This study
pOG-VW-106	Regulatory region of *icmVW* in pUC18	This study
pOG-W-111	Regulatory region of *icmW* in pMC1403	This study
pOG-W-124	Regulatory region of *icmW* in pGS-lac-01	This study
pSS-R-1	Regulatory region of *icmR* in pUC18	This study
pSS-R-27	Regulatory region of *icmR* in pMC1403	This study
pUC18	*oriR*(ColE1) MCS Ap^r	53

icm/*dot* gene expression. The mutation in the *relA* gene had no effect on *L. pneumophila* intracellular growth in HL-60-derived human macrophages or *A. castellanii.* A reduction in the expression of only one of nine *icm*::*lacZ* fusions was observed in the *relA* insertion mutant, and a similar result was obtained with an *rpoS* insertion mutant.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and media. The *L. pneumophila* strains used in this work were JR32, a streptomycin-resistant, restriction-negative mutant of *L. pneumophila* Philadelphia-1 which is a wild-type strain in terms of intracellular growth (35); 25D, a mutant that cannot grow intracellularly (22); LM1376, an *rpoS* insertion mutant which is a JR32 derivative (17); and *relA* deletion substitution mutants GS-RelA and GS-RelA2 (two independent isolates of the *relA* mutant, which originated from two different electroporations), which are also JR32 derivatives. The plasmids and primers used in this work are described in Tables 1 and 2, respectively. The bacterial media, plates, and antibiotic concentrations used have been described previously (38).

Construction of plasmid for allelic exchange. Primers relA-F and relA-R (Table 2) were designed by using *L. pneumophila* genome sequence information (http://genome3.cpmc.columbia.edu/~legion/index.html). Primers relA-F and relA-R overlap the *relA* start codon and stop codon, respectively, and they were used to amplify a 2.2-kb DNA fragment containing the *relA* gene; this fragment was cloned into pUC18 digested with *Hinc*II to generate pGS-relA-01. To knock out the *relA* gene, the kanamycin resistance cassette (Pharmacia) was cloned into

TABLE 2. Primers used in this study

Primer	Sequence (5′-3′)
flaA-Up	CAAAAAAGCGCTTTCGGAAC
flaA-Down	GGGATCCCCGTTGATTACTTGAGCCATAATTTTAGTC
icmF-F	GAACAAGGAGCAAGTATTTC
icmF-R	GGATCCCCGTATTGCTCAGTTGTCATTATATTAC
icmJ-F	TAAAGAGAATACCGCTTTACCC
icmJ-R	GGGATCCCCTCGTTGTTGATTATCCGCCAT
icmM-F	TGGTCTCAGGGGGTTGATAG
icmM-R	GGATCCCCCCATGTTTCTCGACTCATTTTTAC
icmP-F	TATATCGATACTCCAATGGCC
icmP-R	GGGATCCCCTTGCTGCTGTTGTTGTGCCAT
icmQ-F	AGCCATGATGAACGTGGTTTC
icmQ-R	GGGATCCCCATCAGTATTATTACCCATTATTAC
icmR-F	CCCTGGATGAGTTAATGTATG
icmR-R	GGGATCCCCCGAGAGTTGATCTTTCATAAC
icmT-F	TCTCCAATGGATTAAGTCCGG
icmT-R	GGGATCCCCTGCTGAAAATCCACCTGCCAT
icmVW-F	GGGATCCCCTGATCCTGATTTCTTTTTCATATT
icmVW-R	GGGATCCCCTTCATGGCTTAAATCAGGCAT
relA-F	ATGGTAAGAGTAAAAGATACGACTCCG
relA-R	CTATAATTGCCTTCTTGCTTCCAG
relA-Up-EI	GCCGGAATTCCATTGGCGCAGATGTTATGG
relA-Down-Bam	CGGGGGATCCTATTGAGCGAACACAGAGCG

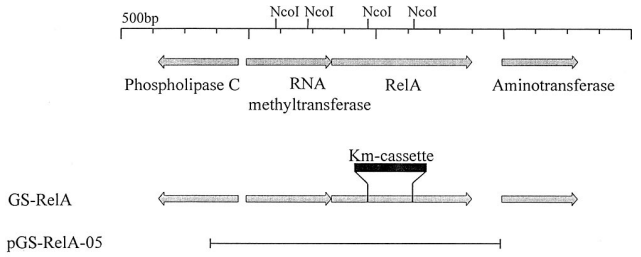

FIG. 1. Linkage map of the *relA* locus. The arrows indicate coding regions. The site of the kanamycin cassette in the GS-RelA deletion substitution mutant is indicated. The restriction enzyme *Nco*I sites used for construction of the *relA* deletion substitution are shown. The line indicates the region covered by the complementing plasmid pGS-RelA-05. (The organization of the genes in the region was based on the incomplete sequence of the *L. pneumophila* genome; therefore, the identities of the three genes near *relA* are tentative.)

it, instead of an internal 721-bp *Nco*I fragment, to generate pGS-relA-01-Km (Fig. 1). Plasmid pGS-relA-01-Km was digested with *Sph*I and *Bam*HI, and after fill-in the insert was cloned into the *Eco*RV site of the allelic exchange vector pLAW344 to form pGS-relA-01-Km-GR. This plasmid was used for allelic exchange, as previously described (38). The resulting strains (GS-RelA and GS-RelA2) contain the first 183 amino acids of the RelA protein, as well as its last 311 amino acids. The internal 240 amino acids were deleted during construction, and the kanamycin resistance cassette was cloned instead of these amino acids. Several isolates were analyzed by PCR to confirm that the right change occurred on the chromosome (data not shown).

Construction of plasmid for RelA complementation. To construct a *relA*-complementing clone, primers relA-Up-EI and relA-Down-Bam were used. A sequence analysis of the upstream region of the *relA* gene showed that an RNA methyltransferase homologous gene was located immediately upstream of this gene (Fig. 1). As we assumed that the two genes form an operon, PCR amplification was used to obtain both genes. Two primers were used to amplify a 3.8-kb DNA fragment that was subsequently digested with *Eco*RI and *Bam*HI and cloned into the same sites in vector pMMB207αb to form pGS-relA-05. This plasmid was used for complementation.

Construction of *lacZ* fusions. The promoterless *lacZ* vector pMC1403 (8) and plasmid pAB1 (36) were used to construct an *L. pneumophila* promoterless *lacZ* vector, pGS-lac-01. Plasmid pAB-1 was digested with *Sac*I and *Xmn*I, and plasmid pMC1403 was digested with *Sac*I and *Apa*I (in both plasmids the *Sac*I site is located in the middle of the *lacZ* gene). The 10-kb *Sac*I-*Xmn*I fragment of pAB-1 containing the pMMB207 vector and part of the *lacZ* gene was ligated with the 2.3-kb *Sac*I-*Apa*I fragment of pMC1403 containing part of the promoterless *lacZ* gene in order to generate pGS-lac-01. pGS-lac-01 did not exhibit a significant level of β-galactosidase activity in either *E. coli* or *L. pneumophila* during the exponential and stationary phases (data not shown), and it was used to construct the *icm*::*lacZ* and *flaA*::*lacZ* fusions described below.

The *icm* genes for which *icm*::*lacZ* fusions were constructed were chosen on the basis of the complementation analysis that was performed previously (32, 37, 38). The location of the upstream primer (F primer) was chosen on the basis of the gene organization of the region, and at least 150 bp upstream from the first methionine of each gene was included. The downstream primer (R primer) was designed to generate an in-frame fusion between the first seven amino acids of the *icm* gene and the *lacZ* reporter gene. In addition, a *lacZ* fusion to the *flaA* gene regulatory region was constructed. To construct the *icm*::*lacZ* and *flaA*::*lacZ* fusions, the regulatory region of the *icmT*, *-R*, *-Q*, *-P*, *-M*, *-J*, *-F*, *-V*, and *-W* and *flaA* genes (in the case of *icmF* the fusion constructed was a fusion to the gene located upstream of it designated 47:8g [13]; these two genes are probably located on the same transcriptional unit) was amplified by PCR with the primers described in Table 2. The fragments generated were cloned into pUC-18 digested with *Sma*I to generate pOG-T-104, pSS-R-1, pKP-Q-1, pOG-P-105, pGS-reg-M1, pOG-J-103, pGS-reg-F1, pOG-VW-106 (for both *icmV* and *icmW*), and pGS-flaA-01, respectively. All of the plasmids were sequenced to confirm that no changes were made during PCR amplification. These plasmids were digested with *Bam*HI (one site was located in the R primer, and one site was located in the vector) and cloned into pMC1403 digested with *Bam*HI to obtain plasmids pOG-T-107, pSS-R-27, pKP-Q-21, pOG-P-108, pGS-reg-M2, pOG-J-109, pGS-reg-F2, pOG-V-110, pOG-W-111, and pGS-flaA-02, respectively. Clones that were found to be blue on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were tested to determine the orientations of their inserts. In all of the blue clones tested only the orientation expected to generate an active fusion was found. These plasmids were digested with *Eco*RI and *Sac*I (the *Eco*RI site was located upstream of the *Bam*HI cloning site into which the regulatory regions were cloned, and the *Sac*I site was located inside the *lacZ* gene) and cloned into pGS-lac-01 digested with the same enzymes to obtain plasmids pOG-T-120, pOG-R-125, pOG-Q-126, pOG-P-121, pGS-reg-M3, pOG-J-122, pGS-reg-F3, pOG-V-123, pOG-W-124, and pGS-flaA-03, respectively. These plasmids were used for experiments with *L. pneumophila* in which the effect of the *relA* insertion mutation on *icm* and *flaA* gene expression was determined relative to expression in the wild-type strain.

Measurement of ppGpp production in *L. pneumophila*. ppGpp production was analyzed by thin-layer chromatography (TLC) essentially as described previously (9). *L. pneumophila* exponential cultures grown in regular AYE (ACES yeast extract) medium were labeled with carrier-free [^{32}P]phosphoric acid (500 μCi ml^{-1}; ICN Pharmaceuticals) for about 6 h before sampling. Samples were obtained during the early stationary phase (optical density at 600 nm [OD_{600}], 2.6), the stationary phase (OD_{600}, 3.1) and the late stationary phase (OD_{600}, 3.6). At each time a 50-μl aliquot was extracted with 50 μl of 13 M formic acid, and 4 μl of extract was applied to a polyethyleneimine-cellulose TLC sheet (Baker) and developed for 40 min with 1.5 M KH_2PO_4 (pH 3.4). The optical densities of nonradioactive cultures grown under the same conditions were determined. The locations of ppGpp and GTP on the TLC autoradiogram were determined by using the results of a previous analysis of ppGpp in *E. coli* (9) and *L. pneumophila* (18) in which the same protocol was used. The relative amounts of ppGpp were determined with a Phosphor-Imager (Fuji BAS1000).

Intracellular growth in HL-60-derived macrophages. Intracellular growth assays with HL-60-derived macrophages were performed like assays described previously (40). Wells of a 24-well microtiter dish containing 2×10^6 differentiated HL-60-derived macrophages were used for infection. *L. pneumophila* cells were added to the wells at a multiplicity of infection of approximately 0.1, and the infected HL-60-derived macrophages were incubated for 1 h at 37°C under 5% CO_2. Then the wells were washed three times, and 0.6 ml of RPMI medium containing 2 mM Gln and 10% normal human serum was added to each well. The supernatant of each well was sampled at intervals of about 24 h, and the numbers of CFU were determined by plating samples on ACES buffered charcoal yeast extract (ABCYE) plates.

Intracellular growth in *A. castellanii*. Intracellular growth assays with *A. castellanii* were performed like assays described previously (41). A total of 1.5×10^5 amoebae in PYG were added to wells of a 24-well microtiter dish, and the amoebae were incubated for 1 h at 37°C so that they could adhere. Then the PYG was aspirated, the wells were washed once with 0.5 ml of warm (37°C) Ac buffer, and 0.5 ml of warm Acanthamoebae buffer (Ac buffer) was added to each well. Then *L. pneumophila* in Ac buffer was added to the wells at a multiplicity of infection of approximately 0.1. The plate was incubated for 30 min at 37°C, and then the Ac buffer was aspirated, the wells were washed three times with 0.5 ml of warm Ac buffer, and 0.6 ml of warm Ac buffer was added to each well. The supernatant in each well was sampled at intervals of about 24 h, and the numbers of CFU were determined by plating samples on ABCYE plates.

Pigmentation measurements. *L. pneumophila* strains were grown on ABCYE plates containing chloramphenicol for 48 h. The bacteria were scraped off the plates and suspended in AYE broth, and the OD_{600} in AYE broth containing chloramphenicol was adjusted to 0.1. Then 44-ml cultures were prepared, and 1.5-ml portions of the cultures were placed into tubes and grown for 14 to 16 h on a roller drum. Starting when the cultures reached an OD_{600} of about 3, triplicate samples were removed at intervals of 2 h, and pigment production and bacterial density were determined. One milliliter of culture was centrifuged for 10 min at 20,000 × *g*, and the supernatant was transferred to another tube. The bacterial pellet was resuspended in 1 ml of M63 medium (31), and a 1/10 dilution was prepared before the OD_{600} was measured. Pigmentation was analyzed by determining the absorbance of the culture supernatant at 550 nm (50).

β-Galactosidase assays. β-Galactosidase assays were performed as described elsewhere (31). *L. pneumophila* strains were grown on ABCYE plates containing chloramphenicol for 48 h. The bacteria were scraped off the plates and suspended in AYE broth, and the OD_{600} was adjusted to 0.1 in AYE broth. The resulting cultures were grown on a roller drum for 17 to 18 h until the OD_{600} was about 3.2 (stationary phase). The assays were performed with 20 or 50 μl of culture, and the substrate for *lacZ* hydrolysis was *o*-nitrophenyl-β-D-galactopyranoside.

Sodium sensitivity. The sodium sensitivities of the wild type (JR32) and a mutant (GS-RelA) were quantified by growing the bacteria for 72 h on ABCYE

plates, scraping the bacteria off the plates, and adjusting the OD_{600} of each preparation to 4. Then eight 10-fold serial dilutions were plated on ABCYE plates containing or not containing 100 mM NaCl. Sodium sensitivity was determined by comparing the numbers of bacteria growing on the plates. Nonvirulent mutant 25D was used as a sodium resistance control strain.

RESULTS

Construction of an *L. pneumophila relA* deletion substitution mutant. Previously, it was proposed that the *relA* gene product is involved in pathogenicity of *L. pneumophila* (18). To test this proposal directly, we decided to construct a deletion substitution in the *relA* gene and test its effect on pathogenicity and *icm* gene expression. Using a BLAST search (2) with the sequences available from the *L. pneumophila* genome project (http://genome3.cpmc.columbia.edu/~legion/index.html), we found the *L. pneumophila relA* homolog. The RelA protein was found to be 44% identical and 63% similar to *E. coli* RelA (the sequence information is based on an incomplete *L. pneumophila* genome sequence, and it might contain errors that change the levels of identity and similarity slightly). Unlike the *E. coli* gene, the *L. pneumophila relA* gene is probably located on the same transcriptional unit together with an RNA methyltransferase gene (Fig. 1). A similar gene organization has been found in other bacteria, such as *Haemophilus influenzae* (15), *Vibrio cholerae* (20), *Pseudomonas aeruginosa* (45). By using the standard allelic exchange procedure (see Materials and Methods) a deletion substitution in the *relA* gene was constructed by cloning the kanamycin resistance cassette instead of the middle part of the *relA* gene (Fig. 1). The strain generated (GS-RelA) grew well in AYE medium and had the same growth rate as wild-type strain JR32, as determined by optical density and CFU analyses (data not shown).

RelA is required for ppGpp accumulation during the stationary phase. As described above, RelA is a ppGpp synthatase that under certain conditions (amino acid starvation, carbon starvation, entry into the stationary phase, etc.) converts GTP to ppGpp. One phenotype of a *relA* knockout strain that was expected was a change in ppGpp accumulation during the stationary phase. To test this hypothesis, we analyzed accumulation of ppGpp in the *relA* knockout strain (GS-RelA) and compared it to accumulation of ppGpp in wild-type strain JR32. As Fig. 2 shows, during the early stationary phase (OD_{600}, 2.6) a small amount of ppGpp was present in the wild-type strain but not in the *relA* mutant. A clearer result was obtained during the stationary phase (OD_{600}, 3.1), when a large amount of ppGpp was present in the wild-type strain but not in the mutant strain. A result similar to the result obtained for the stationary phase was obtained for the late stationary phase (OD_{600}, 3.6) (data not shown). When we analyzed ppGpp accumulation in the mutant strain (GS-RelA) containing the *relA* complementing plasmid (pGS-RelA-05), clear complementation of ppGpp production was observed (Fig. 2). Moreover, a higher level of ppGpp was present in the mutant strain containing the complementing plasmid than in the wild-type strain (a 30% increase in the amount of ppGpp was observed in the early stationary phase, and a 50% increase was observed in the stationary phase). This result was expected because the *relA* gene product was supplied on a plasmid and higher levels of RelA may have been present in the cells. The lack of detectable levels of ppGpp in the *relA* knockout strain clearly indicates that like the *relA* gene product of *E. coli,* the *relA* gene product of *L. pneumophila* is the major ppGpp synthatase.

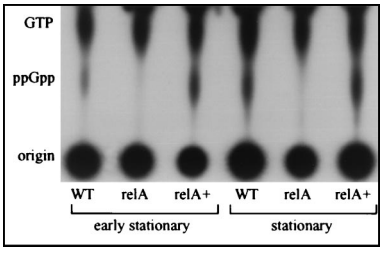

FIG. 2. RelA is required for ppGpp production in *L. pneumophila*. The amounts of ppGpp in wild-type strain JR32 (WT), *relA* mutant strain GS-RelA (relA), and mutant strain GS-RelA complemented with a plasmid (pGS-relA-05) containing the *relA* gene product (relA+) were compared. The nucleotide pools were analyzed during the early stationary phase (OD_{600}, 2.6) and the stationary phase (OD_{600}, 3.1), as described in Materials and Methods. Results similar to the results shown for the stationary phase were obtained for the late stationary phase (OD_{600}, 3.6) (data not shown). The results of one representative experiment of two independent experiments are shown.

RelA influences pigment formation during the stationary phase. *L. pneumophila* forms a brown pigment during the stationary phase of growth, which was shown to be dispensable for intracellular growth (44, 50, 51). Previously, the stationary-phase sigma factor RpoS was found to be dispensable for pigment formation, even though the pigment was produced during the stationary phase (17). Because the *relA* gene product in *E. coli* and other bacteria is known to be involved in regulation of gene expression during the stationary phase (10, 12), we were interested in determining whether pigment production is regulated by *relA*. Therefore, pigment production in wild-type strain JR32 and pigment production in the *relA* mutant GS-RelA during the stationary phase were compared, and a very clear difference was observed (Fig. 3). Compared to the wild-type strain, the *relA* mutant (GS-RelA) began to produce pigment later, and the maximal amount of pigment produced by the mutant after 12 to 14 h during the stationary phase was about one-half the maximal amount produced by the wild type. To confirm that the phenotype observed was due to insertion in the *relA* gene and not to a secondary mutation, a plasmid containing the *relA* gene (pGS-relA-05) was used for complementation. As Fig. 3A shows, when the plasmid containing the *relA* gene product (pGS-RelA-05) was introduced into the GS-RelA strain, pigment began to form at the same time that it began to form in the wild-type strain, and the maximal amount of pigment produced was greater than the maximal amount produced by the wild-type strain. This result was consistent with the result obtained when ppGpp accumulation was determined, which showed that higher levels of ppGpp were produced in the complemented *relA* mutant strain than in the wild-type strain (Fig. 2).

RelA affects *flaA* gene expression during the stationary phase. It is well known that flagellum formation in *L. pneumophila* is correlated with entry into the stationary phase (33), and it has been shown that the flagellum subunit gene (*flaA*) requires the stationary-phase sigma factor for maximal expression (4). In addition, it has been shown previously that overproduction of a truncated form of the *E. coli relA* gene product

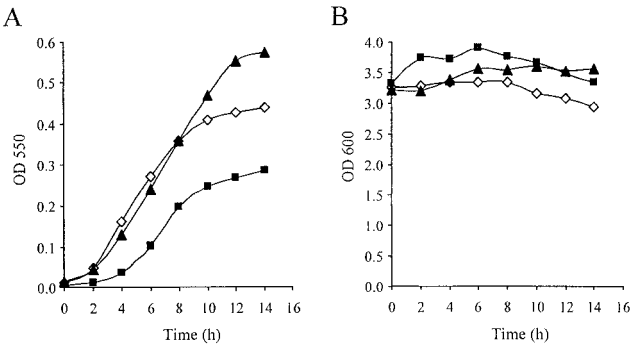

FIG. 3. Pigment production during the stationary phase is dependent on the *relA* gene product. Bacteria were grown to an OD_{600} of 3 (14 to16 h of growth, starting from an OD_{600} of 0.1), and after this (zero time) pigment production (A) and bacterial density (B) were measured at intervals of 2 h. Pigment production was measured by determining the OD_{550} after centrifugation, and bacterial density was measured by determining the OD_{600}. Symbols: ◇, wild-type strain JR32 containing the vector pMMB207αb; ■, RelA mutant strain GS-RelA containing the vector pMMB207αb; ▲, GS-RelA complemented with plasmid pGS-RelA-05. The experiment was done three times, and similar results were obtained in all of the experiments. The largest standard deviation for the pigment production measurements was ±0.015.

in *L. pneumophila* results in expression of the gene encoding the flagellum subunit (*flaA*) (18). To test the effect of the *relA* insertion on expression of the gene coding for the flagellum subunit (*flaA*) during the stationary phase, a translational fusion between *flaA* and the *lacZ* reporter gene was constructed. When this *flaA*::*lacZ* fusion was introduced into the wild-type strain, a very high level of expression (6,607 ± 490 Miller units) was observed during the stationary phase (OD_{600}, 3.2). When the same fusion was introduced into the *relA* mutant (GS-RelA), a dramatic reduction in the β-galactosidase level (1,827 ± 160 Miller units) compared to the level in wild-type strain JR32 was observed (a similar result was obtained during the late stationary phase [OD_{600}, 3.6]). The β-galactosidase levels of these two strains were found to be significantly different ($P > 0.0001$) as determined by a standard *t* test (calculated with data from six independent experiments). This result indicates that the gene coding for the flagellum subunit (*flaA*) is regulated by the *relA* gene product, but it is still expressed at a relatively high level in the absence of *relA.* This result was expected as it is known that flagellum-related genes are regulated by the flagellum sigma factor *rpoF* (21) and by the stationary-phase sigma factor *rpoS* (4), which probably continue to express these genes in the *relA* mutant. To further confirm the effect of the *relA* knockout on flagellum gene expression, the motilities of the *relA* mutant and the wild-type strain were determined microscopically. Between 60 and 70% of the *relA* mutant bacteria were found to be motile during the stationary phase, whereas close to 100% of the wild-type bacteria were motile.

RelA is not required for intracellular growth in eukaryotic hosts. To examine the importance of the *L. pneumophila* RelA protein for intracellular multiplication, the ability of strain GS-RelA to multiply in HL-60-derived macrophages was examined by measuring the change in the number of bacterial CFU in the supernatant of wells containing HL-60-derived macrophages infected with different strains of *L. pneumophila. L. pneumophila* wild-type strain JR32 and mutant strain 25D, which is not able to multiply intracellularly (22), were used as controls. As Fig. 4A shows, the RelA mutant strains (GS-RelA and GS-RelA2) replicated to the same degree as JR32. Therefore, we concluded that RelA is not required for *L. pneumophila* replication in HL-60-derived macrophages. Then the ability of strain GS-RelA to kill HL-60-derived macrophages was tested by using a cytotoxicity assay (38). The results obtained clearly indicate that strain GS-RelA is able to kill HL-60-derived macrophages to the same extent as the wild-type strain (data not shown).

L. pneumophila normally replicates in the environment within protozoan hosts, such as *A. castellanii* (14). Therefore, the abilities of the wild-type and *relA* mutant strains to multiply in amoebae were tested. Strains JR32, 25D, GS-RelA, and GS-RelA2 were used to infect *A. castellanii*, and as Fig. 4B shows, the RelA mutant strains (GS-RelA and GS-RelA2) and the wild-type strain (JR32) replicated to the same extent. We concluded that the *relA* gene product is dispensable for intracellular growth of *L. pneumophila* in the eukaryotic hosts examined.

Wild-type strains of *L. pneumophila* are known to be salt sensitive (11), and mutants that cannot grow intracellularly are salt resistant (35). These two phenotypes were shown to be correlated previously (35). Therefore, we tested the sensitivity to salt (100 mM NaCl) of the *relA* deletion substitution mutant; as expected from the intracellular growth experiments, this strain was found to be salt sensitive to the same extent as the wild-type strain (data not shown).

RelA has a minor effect on *icm* gene expression. To further investigate the possible involvement of the *relA* gene product in intracellular multiplication of *L. pneumophila*, we examined the role of this gene product in *icm* gene expression. Based on the complementation analysis that was performed previously (32, 37, 38), nine *icm*::*lacZ* fusions (*icmT*::*lacZ*, *icmR*::*lacZ*, *icmQ*::*lacZ*, *icmP*::*lacZ*, *icmM*::*lacZ*, *icmJ*::*lacZ*, *icmF*::*lacZ*, *icmV*::*lacZ*, and *icmW*::*lacZ*) were constructed, and the levels

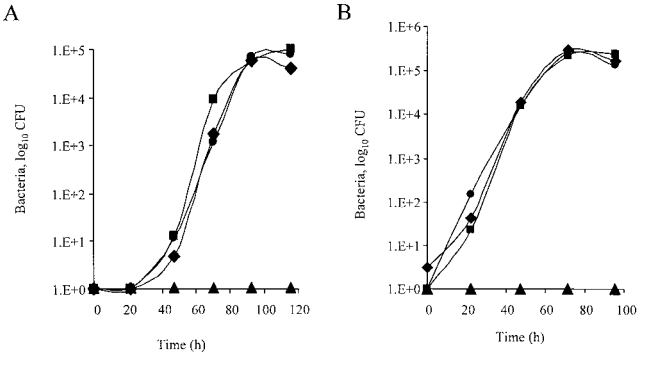

FIG. 4. RelA is dispensable for growth in eukaryotic hosts. Intracellular growth experiments with HL-60-derived human macrophages (A) and with the protozoan host *A. castellanii* (B) were performed as described in Materials and Methods. The experiments were done three times, and similar results were obtained in all of the experiments. Symbols: ■, JR32; ▲,25D; ◆ and ●, independent isolates of the *relA* deletion substitution mutant (GS-RelA and GS-RelA2, respectively).

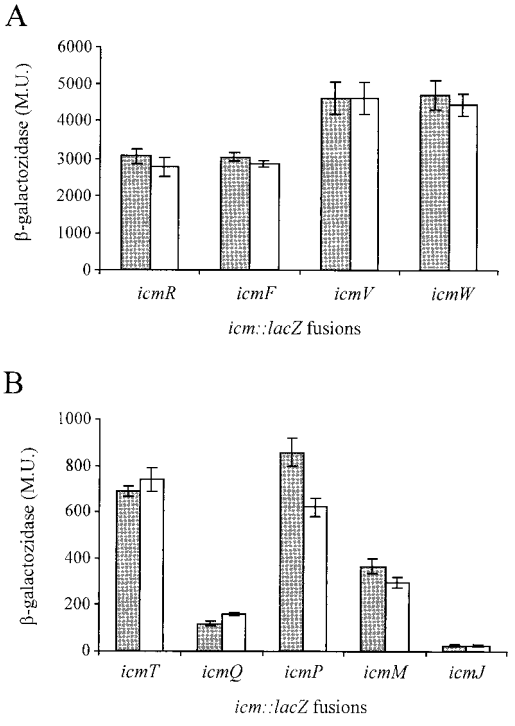

FIG. 5. RelA has a minor effect on expression of one *icm*::*lacZ* fusion. Translational fusions between nine *icm* genes (*icmT*, *icmR*, *icmQ*, *icmP*, *icmM*, *icmJ*, *icmF*, *icmW*, and *icmV*) and the *lacZ* reporter gene were constructed and introduced into wild-type strain JR32 (shaded bars) and RelA insertion mutant GS-RelA (open bars). β-Galactosidase activity was measured as described in Materials and Methods. Four of the *icm*::*lacZ* fusions were found to have high β-galactosidase activities (A), and five had low activities (B). The data are expressed in Miller units (M.U.) and are averages based on at least three different experiments. Expression of *icmP*::*lacZ* in JR32 (858.47 Miller units) and expression of *icmP*::*lacZ* in GS-RelA (621.89 Miller units) were significantly different ($P > 0.0001$), as determined by the standard *t* test. Results similar to the results shown were obtained during the late stationary phase (OD_{600}, 3.6) (data not shown).

of β-galactosidase expression in *relA* mutant strain GS-RelA and wild-type strain JR32 were compared. As Fig. 5 shows, the *icm*::*lacZ* fusions can be divided into two groups based on their levels of expression in the wild-type strain. Four *icm*::*lacZ* fusions (*icmR*::*lacZ*, *icmF*::*lacZ*, *icmV*::*lacZ*, and *icmW*::*lacZ*) exhibited high levels of expression of β-galactosidase, and these fusions were not affected by the *relA* mutation (Fig. 5A). In contrast, five *icm*::*lacZ* fusions (*icmT*::*lacZ*, *icmQ*::*lacZ*, *icmP*::*lacZ*, *icmM*::*lacZ*, and *icmJ*::*lacZ*) exhibited low levels of expression of β-galactosidase, and the level of expression of one of them (*icmP*::*lacZ*) was slightly reduced in the *relA* insertion mutant (Fig. 5B). The level of β-galactosidase expression of the *icmP*::*lacZ* fusion was reduced from 858 to 621 Miller units ($P < 0.0001$). Even with this *lacZ* fusion, the effect was small, and it was probably not enough to influence intracellular multiplication of the *L. pneumophila relA* mutant, as judged from Fig. 4. (It is possible that some minor effects of the *relA* knockout were missed because the *icm*::*lacZ* fusions were located on a plasmid.)

RpoS has a minor effect on *icm* gene expression. The stationary-phase sigma factor RpoS is an additional factor that may be involved in regulation of genes required for intracellular growth of *L. pneumophila*. Unlike the *relA* gene product, the stationary-phase sigma factor has been shown to be required for *L. pneumophila* intracellular growth in *A. castellanii* (17) and to be partially required for growth in murine bone marrow-derived macrophages (4). However, it was also dispensable for intracellular growth in HL-60-derived human macrophages and in THP-1 cells (17). Due to these results and because both RelA and RpoS are involved in expression of genes during the stationary phase, we were interested in determining the involvement of the *rpoS* gene product in *icm* gene expression. The levels of expression of β-galactosidase of the nine *icm*::*lacZ* fusions described above in an *rpoS* mutant strain (LM1376) and wild-type strain JR32 were compared. As Fig. 6 shows, the *rpoS* mutation had a minor effect on expression of the *icm*::*lacZ* fusions, and expression of only two genes was changed. The level of expression of β-galactosidase of the *icmR*::*lacZ* fusion increased from 2,944 to 3,902 Miller units ($P < 0.0001$), and the level of expression of the *icmP*::*lacZ* fusion decreased from 858 to 554 Miller units ($P < 0.0001$).

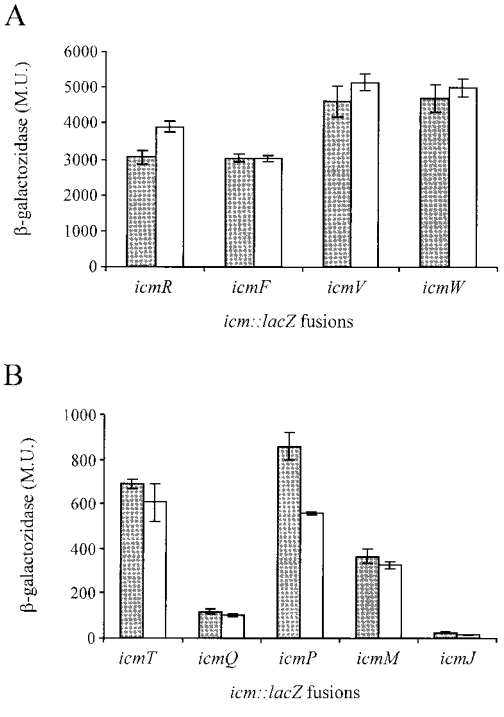

FIG. 6. RpoS has a minor effect on expression of two *icm*::*lacZ* fusions. Translational fusions between nine *icm* genes (*icmT*, *icmR*, *icmQ*, *icmP*, *icmM*, *icmJ*, *icmF*, *icmW*, and *icmV*) and the *lacZ* reporter gene were constructed and introduced into wild-type strain JR32 (shaded bars) and RpoS insertion mutant LM1376 (open bars). β-Galactosidase activity was measured as described in Materials and Methods. Four of the *icm*::*lacZ* fusions were found to have high β-galactosidase activities (A), and five had low activities (B). The data are expressed in Miller units (M.U.) and are averages based on at least three different experiments. Expression of *icmP*::*lacZ* in JR32 (858.47 Miller units) and expression of *icmP*::*lacZ* in LM1376 (554.53 Miller units) were significantly different ($P > 0.0001$), as were expression of *icmR*::*lacZ* in JR32 (2,944.12 Miller units) and expression of *icmR*::*lacZ* in LM1376 (3,902.14 Miller units), as determined by the standard *t* test.

The effect of the *rpoS* mutation on the *icmP*::*lacZ* fusion was similar to the effect of the *relA* mutation on this fusion (Fig. 5B and 6B), and it might have been due to the effect of *relA* on *rpoS* gene expression, as shown in *E. coli* (16). The effect of RpoS and the effect of RelA on *icm* gene expression were found to be minor. These results are in agreement with the data showing that RpoS (17) and RelA (this study) are dispensable for *L. pneumophila* intracellular multiplication in human macrophages, a host in which the *icm* gene products have been shown to be indispensable for intracellular growth (37).

DISCUSSION

During the past several years 24 *icm*/*dot* genes required for intracellular multiplication and host cell killing have been found in *L. pneumophila* (39). Fourteen of these genes have been found to be homologous to the R64 *tra*/*trb* genes involved in conjugation (42), and several of the *L. pneumophila icm*/*dot* genes have been shown to participate in bacterial conjugation (40). Even though 24 *icm*/*dot* genes are known, there is very little information regarding their regulation and the time when they are expressed. Previous reports that addressed this issue indicated that *L. pneumophila* virulence is connected to the stationary phase of growth. It has been shown that the *L. pneumophila rpoS* gene product is required for intracellular growth in the protozoan host *A. castellanii* (17) and is partially required for intracellular growth in murine bone marrow-derived macrophages (4). However, it is dispensable for intracellular growth in HL-60-derived macrophages and THP-1 cells (17). Another report (18) indicated that *L. pneumophila* virulence is coordinated with entry into the stationary phase by ppGpp, the stringent response alarmone. In the same report, several phenotypic traits assumed to be connected with *L. pneumophila* virulence (such as sodium sensitivity, motility, and expression of the flagellum subunit) were shown to be coordinated with ppGpp production (18).

The stringent response in *E. coli* is initiated after starvation for amino acids that leads to inhibition of stable RNA, ribosome, and protein synthesis and for accumulation of ppGpp. This global response is a consequence of binding of uncharged tRNA to ribosomes that activates the *relA* gene product, a guanosine 3′,5′-bispyrophosphate (ppGpp) synthetase. It has been shown that accumulation of ppGpp is involved in several processes, such as activation of the *rpoS* sigma factor in *E. coli* (16, 29), fruiting body formation in *M. xanthus* (19), and antibiotic production in *S. coelicolor* (12). All of these processes appear to be activated during the stationary phase and to depend on the RelA enzymatic activity.

We examined the effect of *L. pneumophila* RelA on gene expression during the stationary phase and on intracellular multiplication. Two independent systems that are known to be expressed during the stationary phase in *L. pneumophila* cultures were found to be affected by the *relA* mutation. Pigment formation was dramatically affected by deletion of the *relA* gene, and only one-half as much pigment was produced in this strain during the stationary phase. This phenotype was complemented with a plasmid containing the *relA* gene, indicating that the *relA* deletion is the only mutation that influences pigment formation in this strain. In addition, expression of the gene coding for the flagellum subunit (*flaA*) was reduced more than threefold in the *relA* mutant compared to the wild-type strain (as determined by a *flaA*::*lacZ* fusion).

When the involvement of RelA in intracellular growth and *icm* gene expression was examined, it became clear that a deletion substitution in *relA* had no effect on intracellular multiplication in the two eukaryotic hosts tested. Moreover, only a minor reduction in β-galactosidase activity was observed in one of the nine *icm*::*lacZ* fusions that were tested. Expression of this fusion (*icmP*::*lacZ*) was reduced to a similar minor extent in an RpoS insertion mutant as well.

In addition to construction of the deletion substitution in *relA*, we also tried to construct a deletion in the *L. pneumophila spoT* homologous gene (the *L. pneumophila spoT* homolog was identified from the *L. pneumophila* genome sequence information by its homology to *E. coli* SpoT gene [52% identity and 70% similarity]). SpoT is the only known *E. coli* ppGpp pyrophosphohydrolase that also possesses ppGpp synthetase activity (9). Several attempts were made to construct such a deletion, but they were not successful. We also tried to construct this deletion in the background of the *relA* mutant strain but had no success (this was the only way in which a *spoT* null strain was constructed in *E. coli* [52]). It is very likely that the *spoT* gene of *L. pneumophila* is essential.

It is known that RelA is the major source of ppGpp in *E. coli*, and as was clearly shown here, this is also the case in *L. pneumophila.* The dramatic decreases in expression of two independent systems that were observed in the *L. pneumophila* RelA mutant probably were due to the lack of ppGpp in the cells during the stationary phase. The ppGpp data indicate that SpoT, by it self, does not produce detectable levels of ppGpp in the conditions examined and that it cannot overcome the deletion in *relA*. The fact that in a bacterial strain the *relA* mutation had a clear effect on ppGpp accumulation, pigment production, and *flaA* gene expression but no effect on intracellular growth clearly indicates that factors other than *relA* control *icm* gene expression.

As described above, the stationary-phase sigma factor RpoS was shown to be involved in *L. pneumophila* intracellular growth in *A. castellanii*, and it has also been suggested that the stringent response system participates together with RpoS in regulation of *L. pneumophila* virulence (4, 18). However, both RpoS and RelA were shown here to have minor effects on *icm*/*dot* gene expression. The *icm*/*dot* genes have been shown to be required for intracellular growth in all eukaryotic hosts examined, including HL-60- and U937-derived human macrophages (37, 49), murine bone marrow-derived macrophages (47), and the protozoan hosts *A. castellanii* (41) and *Dictyostelium discoideum* (43). Taking all of these results together, we believe that it is very likely that some of the virulence factors required for intracellular growth in amoebae are regulated by the *rpoS* sigma factor, but the major system that contributes to *L. pneumophila* intracellular growth (the *icm*/*dot* system) is probably controlled by other regulatory factors. These factors remain to be identified.

ACKNOWLEDGMENTS

We thank Karen Pomeranz and Shai Senderovich for plasmid construction. We are grateful to Howard A. Shuman for carefully reading the manuscript.

This work was supported by a grant from the Israel Science Foun-

dation and by the Tel-Aviv University internal research fund. G. Segal was supported by the Alon fellowship from the Israeli Ministry of Education.

REFERENCES

1. **Abu Kwaik, Y.** 1996. The phagosome containing *Legionella pneumophila* within the protozoan *Hartmannella vermiformis* is surrounded by the rough endoplasmic reticulum. Appl. Environ. Microbiol. **62:**2022–2028.
2. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
3. **Andrews, H. L., J. P. Vogel, and R. R. Isberg.** 1998. Identification of linked *Legionella pneumophila* genes essential for intracellular growth and evasion of the endocytic pathway. Infect. Immun. **66:**950–958.
4. **Bachman, M. A., and M. S. Swanson.** 2001. RpoS co-operates with other factors to induce *Legionella pneumophila* virulence in the stationary phase. Mol. Microbiol. **40:**1201–1214.
5. **Berger, K. H., J. J. Merriam, and R. R. Isberg.** 1994. Altered intracellular targeting properties associated with mutations in the *Legionella dotA* gene. Mol. Microbiol. **14:**809–822.
6. **Bozue, J. A., and W. Johnson.** 1996. Interaction of *Legionella pneumophila* with *Acanthamoeba castellanii*: uptake by coiling phagocytosis and inhibition of phagosome-lysosome fusion. Infect. Immun. **64:**668–673.
7. **Brand, B. C., A. B. Sadosky, and H. A. Shuman.** 1994. The *Legionella pneumophila icm* locus: a set of genes required for intracellular multiplication in human macrophages. Mol. Microbiol. **14:**797–808.
8. **Casadaban, M. J., J. Chou, and S. N. Cohen.** 1980. In vitro gene fusions that join an enzymatically active beta-galactosidase segment to amino-terminal fragments of exogenous proteins: *Escherichia coli* plasmid vectors for the detection and cloning of translational initiation signals. J. Bacteriol. **143:**971–980.
9. **Cashel, M.** 1994. Detection of (p)ppGpp accumulation patterns in *Escherichia coli* mutants, p. 341–356. *In* K. W. Adolph (ed.), Methods in molecular genetics, vol. 3. Academic Press, New York, N.Y.
10. **Cashel, M., D. R. Gentry, V. J. Hernandez, and D. Vinella.** 1994. The stringent response, p. 1458–1496. *In* F. C. Neidhardt (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 1. American Society for Microbiology, Washington, D.C.
11. **Catrenich, C. E., and W. Johnson.** 1989. Characterization of the selective inhibition of growth of virulent *Legionella pneumophila* by supplemented Mueller-Hinton medium. Infect. Immun. **57:**1862–1864.
12. **Chakraburtty, R., and M. Bibb.** 1997. The ppGpp synthetase gene (*relA*) of *Streptomyces coelicolor* A3(2) plays a conditional role in antibiotic production and morphological differentiation. J. Bacteriol. **179:**5854–5861.
13. **Edelstein, P. H., M. A. Edelstein, F. Higa, and S. Falkow.** 1999. Discovery of virulence genes of *Legionella pneumophila* by using signature tagged mutagenesis in a guinea pig pneumonia model. Proc. Natl. Acad. Sci. USA **96:**8190–8195.
14. **Fields, B. S.** 1996. The molecular ecology of legionellae. Trends Microbiol. **4:**286–290.
15. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. F. Tomb, B. A. Dougherty, J. M. Merrick, K. Mckenney, G. Sutton, W. Fitzhugh, C. Fields, G. J. D., J. Scott, R. Shirley, L. I. Liu, A. Glodek, J. M. Kelley, J. F. Weidman, C. A. Phillips, T. Spriggs, E. Hedblom, M. D. Cotton, T. R. Utterback, M. C. Hanna, D. T. Nguyen, D. M. Saudek, R. C. Brandon, L. D. Fine, J. L. Fritchman, J. L. Fuhrmann, N. S. M. Geoghagen, C. L. Gnehm, L. A. Mcdonald, K. V. Small, C. M. Fraser, H. O. Smith, and J. C. Venter.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512.
16. **Gentry, D. R., V. J. Hernandez, L. H. Nguyen, D. B. Jensen, and M. Cashel.** 1993. Synthesis of the stationary-phase sigma factor sigma S is positively regulated by ppGpp. J. Bacteriol. **175:**7982–7989.
17. **Hales, L. M., and H. A. Shuman.** 1999. The *Legionella pneumophila rpoS* gene is required for growth within *Acanthamoeba castellanii*. J. Bacteriol. **181:**4879–4889.
18. **Hammer, B. K., and M. S. Swanson.** 1999. Co-ordination of *Legionella pneumophila* virulence with entry into stationary phase by ppGpp. Mol. Microbiol. **33:**721–731.
19. **Harris, B. Z., D. Kaiser, and M. Singer.** 1998. The guanosine nucleotide (p)ppGpp initiates development and A-factor production in *Myxococcus xanthus*. Genes Dev. **12:**1022–1035.
20. **Heidelberg, J. F., J. A. Eisen, W. C. Nelson, R. A. Clayton, M. L. Gwinn, R. J. Dodson, D. H. Haft, E. K. Hickey, J. D. Peterson, L. Umayam, S. R. Gill, K. E. Nelson, T. D. Read, H. Tettelin, D. Richardson, M. D. Ermolaeva, J. Vamathevan, S. Bass, H. Qin, I. Dragoi, P. Sellers, L. McDonald, T. Utterback, R. D. Fleishmann, W. C. Nierman, and O. White.** 2000. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature **406:**477–483.
21. **Heuner, K., J. Hacker, and B. C. Brand.** 1997. The alternative sigma factor sigma-28 of *Legionella pneumophila* restores flagellation and motility to an *Escherichia coli fliA* mutant. J. Bacteriol. **179:**17–23.
22. **Horwitz, M. A.** 1987. Characterization of avirulent mutant *Legionella pneumophila* that survive but do not multiply within human monocytes. J. Exp. Med. **166:**1310–1328.
23. **Horwitz, M. A.** 1983. Formation of a novel phagosome by the Legionnaires' disease bacterium (*Legionella pneumophila*) in human monocytes. J. Exp. Med. **158:**1319–1331.
24. **Horwitz, M. A.** 1983. The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. J. Exp. Med. **158:**2108–2126.
25. **Horwitz, M. A.** 1984. Phagocytosis of the Legionnaires' disease bacterium (*Legionella pneumophila*) occurs by a novel mechanism: engulfment within a pseudopod coil. Cell **36:**27–33.
26. **Horwitz, M. A., and F. R. Maxfield.** 1984. *Legionella pneumophila* inhibits acidification of its phagosome in human monocytes. J. Cell Biol. **99:**1936–1943.
27. **Horwitz, M. A., and S. C. Silverstein.** 1980. Legionnaires' disease bacterium (*Legionella pneumophila*) multiplies intracellularly in human monocytes. J. Clin. Investig. **60:**441–450.
28. **Komano, T., T. Yoshida, K. Narahara, and N. Furuya.** 2000. The transfer region of IncI1 plasmid R64: similarities between R64 *tra* and *Legionella icm*/*dot* genes. Mol. Microbiol. **35:**1348–1359.
29. **Lange, R., D. Fischer, and R. Hengge-Aronis.** 1995. Identification of transcriptional start sites and the role of ppGpp in the expression of *rpoS*, the structural gene for the sigma S subunit of RNA polymerase in *Escherichia coli*. J. Bacteriol. **177:**4676–4680.
30. **Marra, A., S. J. Blander, M. A. Horwitz, and H. A. Shuman.** 1992. Identification of a *Legionella pneumophila* locus required for intracellular multiplication in human macrophages. Proc. Natl. Acad. Sci. USA **89:**9607–9611.
31. **Miller, J. H.** 1972. Experiments in molecular biology. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Purcell, M. W., and H. A. Shuman.** 1998. The *Legionella pneumophila icmGCDJBF* genes are required for killing of human macrophages. Infect. Immun. **66:**2245–2255.
33. **Rowbotham, T. J.** 1986. Current views on the relationships between amoebae, legionellae and man. Isr. J. Med. Sci. **22:**678–689.
34. **Rowbotham, T. J.** 1980. Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. J. Clin. Pathol. **33:**1179–1183.
35. **Sadosky, A. B., L. A. Wiater, and H. A. Shuman.** 1993. Identification of *Legionella pneumophila* genes required for growth within and killing of human macrophages. Infect. Immun. **61:**5361–5373.
36. **Sadosky, A. B., J. W. Wilson, H. M. Steinman, and H. A. Shuman.** 1994. The iron superoxide dismutase of *Legionella pneumophila* is essential for viability. J. Bacteriol. **176:**3790–3799.
37. **Segal, G., M. Purcell, and H. A. Shuman.** 1998. Host cell killing and bacterial conjugation require overlapping sets of genes within a 22-kb region of the *Legionella pneumophila* genome. Proc. Natl. Acad. Sci. USA **95:**1669–1674.
38. **Segal, G., and H. A. Shuman.** 1997. Characterization of a new region required for macrophage killing by *Legionella pneumophila*. Infect. Immun. **65:**5057–5066.
39. **Segal, G., and H. A. Shuman.** 1998. How is the intracellular fate of the *Legionella pneumophila* phagosome determined? Trends Microbiol. **6:**253–255.
40. **Segal, G., and H. A. Shuman.** 1998. Intracellular multiplication and human macrophage killing by *Legionella pneumophila* are inhibited by conjugal components on IncQ plasmid RSF1010. Mol. Microbiol. **30:**197–208.
41. **Segal, G., and H. A. Shuman.** 1999. *Legionella pneumophila* utilizes the same genes to multiply within *Acanthamoeba castellanii* and human macrophages. Infect. Immun. **67:**2117–2124.
42. **Segal, G., and H. A. Shuman.** 1999. Possible origin of the *Legionella pneumophila* virulence genes and their relation to *Coxiella burnetii*. Mol. Microbiol. **33:**669–670.
43. **Solomon, J. M., A. Rupper, J. A. Cardelli, and R. R. Isberg.** 2000. Intracellular growth of *Legionella pneumophila* in *Dictyostelium discoideum*, a system for genetic analysis of host-pathogen interactions. Infect. Immun. **68:**2939–2947.
44. **Steinert, M., H. Engelhard, M. Flugel, E. Wintermeyer, and J. Hacker.** 1995. The Lly protein protects *Legionella pneumophila* from light but does not directly influence its intracellular survival in *Hartmannella vermiformis*. Appl. Environ. Microbiol. **61:**2428–2430.
45. **Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. Wong, Z. Wu, and I. T. Paulsen.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature **406:**959–964.
46. **Swanson, M. S., and R. R. Isberg.** 1995. Association of *Legionella pneumophila* with the macrophage endoplasmic reticulum. Infect. Immun. **63:**3609–3620.
47. **Vogel, J. P., H. L. Andrews, S. K. Wong, and R. R. Isberg.** 1998. Conjugative transfer by the virulence system of *Legionella pneumophila*. Science **279:**873–876.

48. **Vogel, J. P., and R. R. Isberg.** 1999. Cell biology of *Legionella pneumophila*. Curr. Opin. Microbiol. **2:**30–34.
49. **Wiater, L. A., K. Dunn, F. R. Maxfield, and H. A. Shuman.** 1998. Early events in phagosome establishment are required for intracellular survivel of *Legionella pneumophila*. Infect. Immun. **66:**4450–4460.
50. **Wiater, L. A., A. B. Sadosky, and H. A. Shuman.** 1994. Mutagenesis of *Legionella pneumophila* using Tn*903*dll*lacZ*: identification of a growth-phase-regulated pigmentation gene. Mol. Microbiol. **11:**641–653.
51. **Wintermeyer, E., M. Flugel, M. Ott, M. Steinert, U. Rdest, K. H. Mann, and J. Hacker.** 1994. Sequence determination and mutational analysis of the *lly* locus of *Legionella pneumophila*. Infect. Immun. **62:**1109–1117.
52. **Xiao, H., M. Kalman, K. Ikehara, S. Zemel, G. Glaser, and M. Cashel.** 1991. Residual guanosine 3′,5′-bispyrophosphate synthetic activity of *relA* null mutants can be eliminated by *spoT* null mutations. J. Biol. Chem. **266:**5980–5990.
53. **Yanish-Perron, C., J. Viera, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.