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Icm/Dot-Independent Entry of *Legionella pneumophila* into Amoeba and Macrophage Hosts

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***Legionella pneumophila*, the causative agent of Legionnaires' disease, expresses a type IVB secretion apparatus that translocates bacterial proteins into amoeba and macrophage hosts. When stationary-phase cultures are used to infect hosts, the type IVB apparatus encoded by the *icm*/*dot* genes is required for entry, delay of phagosome-lysosome fusion, and intracellular multiplication within host cells. Null mutants with mutations in *icm*/*dot* genes are defective in these phenotypes. Here a new model is described in which hosts are infected with stationary-phase cultures that have been incubated overnight in pH 6.5 buffer. This model is called Ers treatment because it enhances the resistance to acid, hydrogen peroxide, and antibiotic stress beyond that of stationary-phase cultures. Following Ers treatment entry into amoeba and macrophage hosts does not require *dotA*, which is essential for *Legionella* virulence phenotypes when hosts are infected with stationary-phase cultures, *dotB*, *icmF*, *icmV*, or *icmX*. Defective host entry is also suppressed for null mutants with mutations in the KatA and KatB catalase-peroxidase enzymes, which are required for proper intracellular growth in amoeba and macrophage hosts. Ers treatment-induced suppression of defective entry is not associated with increased bacterial adhesion to host cells or with morphological changes in the bacterial envelope but is dependent on protein expression during Ers treatment. By using proteomic analysis, Ers treatment was shown to induce a protein predicted to contain eight tetratricopeptide repeats, a motif previously implicated in enhanced entry of *L. pneumophila*. Characterization of Ers treatment-dependent changes in expression is proposed as an avenue for identifying *icm*/*dot*-independent factors that function in the entry of *Legionella* into amoeba and macrophage hosts.**

Legionella pneumophila, the causative agent of Legionnaires' disease, is a facultative intracellular pathogen found in aquatic environments as planktonic cells, in biofilms, and as an intracellular parasite of amoebae (14, 61, 62). When internalized by alveolar macrophages (54), *L. pneumophila* resides in specialized phagosomes that are delayed in fusion with lysosomes and are permissive to bacterial replication (78, 81). Formation of the specialized phagosomes is attributed to proteins secreted into the host (16, 23, 48, 53) by a type IVB secretion apparatus encoded by the *icm*/*dot* loci (intracellular multiplication/defective organelle trafficking) (63, 69, 73, 80). Null mutants with mutations in *icm*/*dot* genes are defective in entry into macrophage and amoeba hosts, intracellular multiplication, delay of phagosome-lysosome fusion, and host cell killing (11, 39, 63, 66, 70, 80). Involvement of non-*icm*/*dot* factors in *Legionella* virulence is implicated by mutations outside the *icm*/*dot* loci associated with defective intracellular multiplication and host cell killing. Such mutations implicate type II secretion, iron sequestration, pilin synthesis, hydrogen peroxide decomposition, amino acid biosynthesis, and sugar transport (5, 17, 80). Delayed fusion of *Legionella*-containing phagosomes with lysosomes requires type IVB secretion. However, type IVB secretion is not required for the delayed acquisition of certain lysosomal markers, which occurs in phagosomes containing mutants with mutations in *dotA* and *dotB*, which are considered essential for type IVB secretion (43, 79). These data implicate the presence of *icm*/*dot*-independent virulence factors, which are poorly defined.

The importance of environmental stress in the transition of *L. pneumophila* from environmental bacterium to intracellular pathogen is supported by several lines of evidence. Nutrient, thermal, and osmotic stresses are imposed in the environmental reservoirs from which Legionnaires' disease is spread, including water cooling towers, hot water tanks, and evaporative condensers of air conditioning systems. Increased resistance to antibiotics, to biocides, and to acid, H_2O_2, osmotic, and thermal stresses following growth within amoebae (19, 20, 62) suggests that stress is imposed by environmental amoeba hosts (2, 29, 32, 38, 74, 80) belonging to the genera *Acanthamoeba* and *Hartmannella* (14, 62). Stress genes are induced when *Legionella* is grown in amoebae or in macrophage hosts (1, 2, 59; M. Miyaki, T. Fukui, Y. Imai, and H. A. Shuman, Abstr. 103th Gen. Meet. Am. Soc. Microbiol., abstr. B-013, 2003). Participants in the oxidative stress response, the KatA and KatB catalase-peroxidase enzymes, are required for optimal intracellular multiplication in macrophages (5). Finally, the stress of growth to the stationary phase endows *L. pneumophila* with a full panel of virulence traits compared to rapidly dividing exponential cultures, which are essentially avirulent (15).

We report here a new model for studying the effect of stress

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or features	Reference
Strains		
JR32	Salt-sensitive isolate of AM511 (Philadelphia-1 Sm^r r^-m^+)	66
katA (PB126)	JR32 *katA*::Gm^r	6
katB (PB117)	JR32 *katB*::Ω Cm^r	7
icmS (GS3001)	JR32 *icmS3001*::Km	68
icmP (GS3002)	JR32 *icmP3002*::Km	68
icmX (LELA921)	JR32 *icmX921*::Tn*903*dII*lacZ*	66
icmF (LELA1275)	JR32 *icmF1275*::Tn*903*dII*lacZ*	66
icmV (LELA1747)	JR32 *icmV1747*::Tn*903*dII*lacZ*	66
dotB (LELA2883)	JR32 *dotB2883*::Tn*903*dII*lacZ*	66
dotA (LELA3118)	JR32 *dotA3118*::Tn*903*dII*lacZ*	66
icmS complemented	GS3001/pGS-Lc-37-14	71
icmP complemented	GS3002/pGS-Lc-34-14	71
icmF complemented	LELA1275/pGS-Lc-55-14	71
katA complemented	PB126/pMMB207αB-Km-14::*katA*	6
Plasmids		
pMV206.hyg	BspHI to SmaI hygromycin resistance cassette from p16R1 in pMV206 with Km^r resistance cassette removed	33
pJN105	pBBR1MCS-5 with *araC* and P_{BAD} regions before the polylinker, Gm^r	55
pJN105-hygro-GFP	pJN105 with hygromycin resistance and GFP	This study
pGS-Lc-37-14	*icmTS* in pMMB207αB-Km-14	71
pMMB207	RSF1010 derivative, IncQ, *oriT*, $lacI^q$, P*tac*, Cm^r	52
pGS-Lc-34-14	*icmPO* in pMMB207αB-Km-14	71
pGS-Lc-55-14	*icmF* and *tphA* in pMMB207αB-Km-14	71
pMMB207αB-Km-14	pMMB207, *lacZ*α, MCS, with *mobA*::Km^r	70
pMMB207αB-Km-14::*katA*	*katA* in BamHI site of pMMB207αB-Km-14 opposite the direction of P*tac*	6
pGS-GFP-04	*gfp* in pMMB207	39

on entry of *L. pneumophila* into amoebae and macrophages: overnight incubation of stationary-phase cultures in a phosphate-saline buffer. The model is called Ers treatment (enhanced resistance to stress) because it enhances resistance of stationary cultures to acid, H_2O_2, and antibiotic stresses. Ers treatment suppresses the defective entry into amoeba and macrophage hosts of null mutants with mutations in *dotA* and *dotB*, other *icm*/*dot* genes, and *katA* and *katB* genes. Since the *icm*/*dot* genes are not required for host entry of Ers-treated *Legionella*, *icm*/*dot*-independent entry factors are implicated in the Ers treatment model. Thus, analysis of Ers treatment-induced gene expression may be an avenue for identifying *icm*/*dot*-independent factors involved in the entry of *Legionella* into eukaryotic hosts.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All *L. pneumophila* mutants were derived from serotype 1 wild-type strain JR32 (66, 83) (Table 1). *Legionella* strains were revived from frozen storage on charcoal–*N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES)-buffered pH 6.9 yeast extract agar plates and then cultured in ACES-buffered pH 6.9 yeast extract (AYE) broth (27, 40). Exponential- and stationary-phase cultures were grown to optical densities at 600 nm of 0.5 to 0.9 and >2.5, respectively (15). When required, chloramphenicol, hygromycin sulfate, and gentamicin sulfate were present at concentrations of 5, 100, and 10 μg/ml, respectively. Ers-treated cultures were prepared by resuspension of stationary-phase cultures in *Acanthamoeba castellanii* buffer (AC buffer) (50), followed by incubation for 16 to 19 h. AC buffer contained 4 mM magnesium sulfate, 0.4 mM calcium chloride, 3.4 mM sodium citrate, 0.051 mM ferrous ammonium sulfate, 2.5 mM sodium monohydrogen phosphate, and 2.5 mM potassium dihydrogen phosphate, and the pH was adjusted to 6.5 with HCl. For recombinant construction we used *Escherichia coli* strain DH5α cultured in Luria-Bertani medium (75) with chloramphenicol, hygromycin sulfate, and gentamicin sulfate at concentrations of 25, 150, and 5 μg/ml, respectively. All cultures were incubated at 37°C with aeration. Bacterial viability was determined by the LIVE/DEAD BacLight technique (Molecular Probes, Eugene, Oreg.).

Amoeba and macrophage lines and culture conditions. *A. castellanii* ATCC 30234 was cultured at 28°C in peptone yeast extract glucose (PYG) medium, as described previously (5, 39, 71). The HL60 monocyte cell line was maintained in RPMI medium supplemented with 2 mM glutamine, 10% heat-inactivated fetal bovine serum, and penicillin-streptomycin (5,000 U/ml) at 37°C in a humidified atmosphere containing 5% CO_2 (39, 71).

Growth of *L. pneumophila* in *A. castellanii*. For growth of *L. pneumophila* in *A. castellanii* (39, 71), adherent amoebae were fed with fresh PYG medium the previous day in a 75-cm^2 tissue culture flask. The PYG medium was removed from the flask, 25 ml of AC buffer was added, and stationary-phase wild-type or *katA* null mutant *L. pneumophila* cells were added at a multiplicity of infection (MOI) of 3 to 5. After incubation for 1 h at 37°C, the amoeba monolayer was washed twice and incubated for 48 h at 37°C with 25 ml of fresh AC buffer. The AC buffer, containing *Legionella* released following intracellular multiplication and lysis of the amoeba hosts, was removed. Fresh AC buffer was added, and adherent amoebae containing intracellular bacteria were dislodged (71) and recovered by centrifugation. The amoeba pellet was resuspended in AC buffer and RPMI medium containing glutamine and normal human serum for stress and HL60 entry experiments, respectively, and amoebae were lysed by repeated aspiration through a 30-gauge needle (20, 50). Amoeba debris was removed by centrifugation (1 min at 150 × *g*) (20), and the supernatant containing amoeba-grown *L. pneumophila* was used immediately for stress or entry experiments.

Plasmids. Transformation of *L. pneumophila* with plasmids (Table 1) expressing hygromycin resistance was accomplished by natural competence (51, 77) by using a protocol described previously (51). All other plasmids were transformed by electroporation by using a previously described protocol (18), modified as follows. Cultures of *L. pneumophila* in AYE broth (optical density at 600 nm, 0.4 to 1.0) were centrifuged and resuspended in cold 10% glycerol three times, the last time at a ratio of 2 ml of 10% glycerol per liter of culture. Electroporation was performed in 0.2-cm cuvettes with a Gene Pulser (Bio-Rad, Hercules, Calif.) set at 25 μF and 2.5 kV, and this was followed by 4 to 5 h of outgrowth in AYE broth at 37°C before plating. To perform immunofluorescence entry assays with complemented *L. pneumophila* mutants, it was necessary to construct a green fluorescent protein (GFP)-expressing plasmid compatible with the IncQ plasmids conventionally used for complementation. The pBBR1MCS-5 derivative of the *Bordetella bronchiseptica* BHR plasmid has a pBBR/BHR replicon (45) that is maintained in *L. pneumophila* (47, 48) and is compatible with IncP, IncW, IncQ, and IncRi incompatibility groups (45). PCR primers with BglII sites at their 5′ ends were used to amplify the region outside the gentamicin cassette in

pJN105, a derivative of pBBR1MCS-5 with *araC* and P_{BAD} regions before the polylinker (55). The PCR fragment was digested with BglII, blunted with the Klenow fragment, and ligated to the blunted DdeI-DdeI hygromycin resistance fragment from pMV206.hygro (33) to form pJN105-hygro. The P*tac*-GFP fragment of pGS-GFP04 (39) was amplified by PCR by using primers with NheI sites at their 5′ ends. After digestion with NheI, this fragment was ligated with NheI-digested pJN105-hygro to form pJN105-hygro-GFP. To our knowledge, hygromycin has not previously been reported to be a selectable marker in *L. pneumophila*.

Acid and hydrogen peroxide stress treatments. *L. pneumophila* cultures (exponential, stationary phase, or Ers treated stationary phase) were washed, resuspended in AC buffer, and diluted to the desired cell concentration by assuming that an optical density at 600 nm of 0.3 corresponded to 5×10^8 CFU/ml (39). For acid treatment, 0.5 ml of a bacterial suspension was rapidly mixed with 0.5 ml of 40 mM citric acid in AC buffer to obtain a final pH of 3.0. For H_2O_2 treatment, H_2O_2 was added to 1 ml of a bacterial suspension to a final concentration of 1 mM. After incubation at 37°C with aeration for the desired time, aliquots were diluted into AC buffer and then plated on CYE agar for colony counting. Acid and H_2O_2 stress experiments with wild-type strain JR32 and the *katA* mutant were performed three or more times. Acid stress experiments with *icmF*, *icmP*, and *icmS* mutants were performed two or more times.

Immunofluorescence assay for entry of *Legionella* into *A. castellanii* and HL60-derived macrophages. For the immunofluorescence assay for entry of *Legionella* into *A. castellanii* (39), adherent *A. castellanii* cells were resuspended in PYG medium to a concentration of 1×10^7 cells/ml, and then 0.5 ml was added to wells of a 24-well tissue culture plate containing glass coverslips (diameter, 12 mm). After incubation for 1 h at 28°C in a humidified atmosphere, the PYG medium was removed, and the amoeba monolayer was washed three times with AC buffer. To each well 0.5 ml of AC buffer and an *L. pneumophila* culture in AC buffer were added to obtain an MOI of 3 to 5. The tissue culture plate was centrifuged at room temperature ($700 \times g$, 10 min) to synchronize the infection and then incubated for 30 min at 28°C in a humidified atmosphere. Subsequent washing, formaldehyde fixation, and incubation with rhodamine-conjugated rabbit anti-*L. pneumophila* serotype 1 antibody (m-Tech, Atlanta, Ga.) were performed as described previously (39). Coverslips were inverted and mounted on microscopy slides by using Fluoromount G mounting medium (Southern Biotechnology Associates, Birmingham, Ala.).

HL60 cells were resuspended in RPMI-glutamine-PennStrep medium without fetal bovine serum to a concentration of 3×10^6 cells/ml, and 0.5 ml was added to wells of a 24-well tissue culture plate containing glass coverslips. After incubation for 1 h at 37°C in a humidified 5% CO_2 atmosphere, the medium was removed, and the monolayer was incubated for 20 to 24 h in RPMI-glutamine-PennStrep medium with 10% fetal bovine serum containing 20 nM phorbol 12-myristate 13-acetate to promote differentiation into macrophages. The monolayer was then washed three times with medium without PennStrep or phorbol 12-myristate 13-acetate. Then to each well 0.5 ml of RPMI-glutamine medium containing 10% normal human AB serum (Gemini Bioproducts, Woodland, Calif.) was added, followed by *L. pneumophila* in the same medium at an MOI of 100. The tissue culture plate was centrifuged for 10 min at room temperature and then incubated for 30 min at 37°C. Subsequent washing, formaldehyde fixation, incubation with rhodamine-conjugated anti-*L. pneumophila* antibody, and mounting were performed as described above for the fluorescence-based *A. castellanii* entry experiments (39).

The *L. pneumophila* strains used for the immunofluorescence assay contained one of two plasmids expressing GFP. For strains harboring plasmid pGS-GFP-04 (39), the *Legionella* medium contained chloramphenicol and 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). For strains harboring plasmid pJN105-hygro-GFP, the medium contained hygromycin and 0.5 mM IPTG. Whenever tested, entry with the pGS-GFP-04 (IncQ) GFP-expressing plasmid and entry with the pJN105-hygro-GFP (pBBR/BHR) GFP-expressing plasmid were identical. Each amoeba or macrophage entry experiment was performed in duplicate. In addition, for amoeba entry, duplicate experiments were performed three or more times with the JR32, *katA*, *icmF*, *icmP*, *icmS*, and *dotA* strains. Duplicate macrophage entry experiments were performed two or more times with the JR32, *katA*, *icmF*, *icmP*, *icmX*, and *dotA* strains.

Fluorescence microscopy. For fluorescence microscopy (39), microscope slides with immunofluorescent samples were observed at a magnification of ×90 by using HiQ band-pass GFP and CY3/rhodamine filters (Chroma Technology Corp., Brattleboro, Vt.) with an Olympus 1X70 fluorescence microscope. Only *L. pneumophila* cells that are external or adherent to the host cell surface are accessible to rhodamine-conjugated anti-serotype 1 antibody; these cells are green with the GFP filter and red with the rhodamine filter. *Legionella* cells within a host cell are not accessible to the antibody. Internalized bacteria were defined as the bacteria within the confines of a host cell envelope that were green with the GFP filter and not visible with the rhodamine filter. Microscope slides could be stored in the dark at 4°C for 2 weeks prior to observation with the fluorescence microscope without quantitative changes in the phagocytic index (number of bacteria internalized per 50 host cells).

Gentamicin protection assay of entry into HL60-derived macrophages. For the gentamicin protection assay of entry into HL60-derived macrophages (39), HL60 monocytes (1.5×10^6 cells/well) were plated, differentiated, washed, and incubated with *L. pneumophila* cultures at an MOI 100 as described above for the immunofluorescence method, except that no glass coverslips were added to the 24-well tissue culture plates. Following 10 min of synchronizing centrifugation and an additional 30 min of incubation at 37°C, the HL60 monolayers were washed twice with phosphate-buffered saline and then incubated for 40 min at 37°C with RPMI-glutamine medium with 100 μg of gentamicin per ml to kill external, adherent *Legionella*. The monolayers were washed twice with phosphate-buffered saline and lysed for 30 min with 0.5 ml of water to release internalized bacteria. Aliquots of lysates were plated on CYE agar for colony counting.

Negative-stain electron microscopy. Cultures of the *L. pneumophila dotA* and *icmS* null mutants in AYE broth or after Ers treatment were transferred to Formvar- and carbon-coated grids and negatively stained with 1% phosphotungstic acid or 1% ammonium molybdate. The grids were blotted dry and immediately observed with a JEOL 1200 EX transmission electron microscope at 80 kV. Images for different strains and culture conditions were compared by measuring cell width and length, by observing features of the cell periphery from stain pooled along the cell edges, and by observing the speckling over the cell body from stain pooled in involutions on the cell surface.

Two-dimensional gel electrophoresis. Cell extracts were prepared from broth-grown stationary-phase cultures and Ers-treated stationary-phase cultures by resuspending bacteria in 25 mM potassium phosphate (pH 7.1) containing 1 mM EDTA and 1× Complete EDTA-free protease inhibitor cocktail (Boehringer, Indianapolis, Ind.), followed by sonication (76) and centrifugation at $15{,}000 \times g$ for 40 min. Supernatant proteins were precipitated by phenol-ethyl ether extraction (67) and then dissolved at a concentration of ~8 mg ml^{-1} in isoelectric focusing buffer containing 9 M urea, 2% Triton X-100, 2% dithioerythritol, 0.5% IPG buffer, and pH 4-7L Immobiline strips (Amersham Biosciences, Piscataway, N.J.). The pH 4-7L Immobiline strips (13 cm) were hydrated with a solution containing ~2 mg of protein for 14 h at 20°C and subjected to isoelectric focusing in an IPGphor apparatus (Amersham Biosciences), typically for a total of 40,000 V · h. After isoelectric focusing, each strip was equilibrated with 10 ml of 50 mM Tris HCl (pH 8.8)–6 M urea–30% glycerol–2% sodium dodecyl sulfate (SDS)–2% dithioerythritol for 40 min at room temperature and then incubated for an additional 15 min after addition of iodoacetamide to a final concentration of 2.5%. The strips were then placed in 0.5% agarose on top of 10% acrylamide–SDS gels. After electrophoresis, the gels were stained with 0.05% Coomassie brilliant blue in 7% acetic acid–50% methanol, destained in 7% acetic acid–50% methanol, and then soaked overnight in water and stored at 4°C until they were used for protein identification.

Protein identification by mass spectrometry. Spots of interest that were cut from acrylamide gels following two-dimensional electrophoresis were washed once with 25% acetonitrile in 25 mM Tris HCl (pH 8.5) and twice with 50% acetonitrile in 25 mM Tris HCl (pH 8.5) and then dried under a vacuum and stored at −20°C. For trypsin digestion, each gel spot was incubated overnight at 32°C in 8 μl of sequencing grade modified trypsin (0.01 mg ml^{-1} in 25 mM Tris HCl [pH 8.5]; Promega, Madison Wis.) and 15 μl of water. The peptides in the digestion buffer plus those extracted from the gel with 2% trifluoroacetic acid in 50% acetonitrile were dried under a vacuum and then dissolved in a 4-hydroxy-α-cyanocinnamic acid matrix solution (10 mg ml^{-1} in 0.1% trifluoroacetic acid–50% acetonitrile) and applied to a matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometer sample plate. The masses of tryptic peptides in digestion mixtures were determined by MALDI-TOF mass spectrometry with a Voyager-DE mass spectrometer (PerSeptive Biosystems, Inc. Farmingham, Mass.). Proteins in gel spots were identified by matching experimentally determined masses with masses predicted from open reading frames (ORFs) in the *L. pneumophila* strain JR32 genome (http://genome3.cpmc.columbia.edu/~legion/index.html) by using the Peptide Search Program (http://www.narrador.embl-heidelberg.de/GroupPages/PageLink/peptidesearchpage.html). Unambiguous matches were established by considering the number of peptides matched, the percentage of the ORF covered, the matched uncleaved peptides, and the agreement between the experimental and predicted masses and the isoelectric points for the S-acetamidomethylated polypeptide.

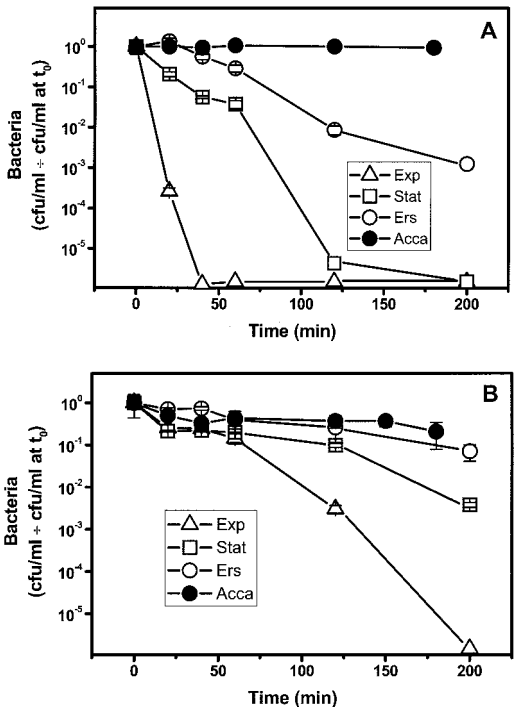

FIG. 1. Effect of growth conditions on stress resistance of wild-type *L. pneumophila.* Exponential-phase (Exp), stationary-phase (Stat), or Ers-treated stationary-phase (Ers) cultures or bacteria recovered from *A. castellanii* following intracellular multiplication (Acca) were challenged with pH 3 (A) or 1 mM H_2O_2 (B) and titrated after different times (mean ± standard deviation). The points plotted on the *x* axis are the values when there were no CFU.

RESULTS

Intra-amoebic growth enhances resistance of wild-type *L. pneumophila* to acid and H_2O_2 stresses. Intracellular multiplication of *L. pneumophila* in environmental amoebae is integral to the spread of Legionnaires' disease (3, 8, 74, 80). We examined if intracellular multiplication in *A. castellanii*—known to enhance entry of *L. pneumophila* into macrophage and amoeba hosts (19, 20)—enhanced the resistance of *Legionella* stationary-phase cultures used to infect amoebae to H_2O_2 and acid stresses. Since *Legionella* is likely to encounter reactive oxygen species following phagocytosis (5, 35, 41, 46) and to encounter acid stress when *Legionella*-containing phagosomes ultimately fuse with lysosomes (78), these stresses are physiologically relevant.

Amoeba-grown *Legionella* maintained an approximately constant titer after challenge by pH 3 (Fig. 1A) or 1 mM H_2O_2 (Fig. 1B). In contrast, the titers of broth-grown stationary-phase cultures decreased by more than 5 orders of magnitude and by 2 orders of magnitude, respectively. Exponential-phase cultures were considerably less resistant to acid and H_2O_2 stresses than stationary-phase cultures (Fig. 1). This cross-resistance of stationary-phase cultures to stress, initially observed in *E. coli* (42), is also found in *L. pneumophila* (4, 36). Wild-type *L. pneumophila* strain AA100 showed increases in resistance to acid, H_2O_2, osmotic, and thermal stresses in bacteria isolated after intracellular multiplication in *Hartmannella vermiformis* compared with the resistance of exponential-phase broth cultures (2). Our demonstration of increased stress resistance following intra-amoebic growth is in qualitative agreement with the results of *H. vermiformis* studies (2). However, a quantitative comparison was not possible due to differences in the stress resistance of the stationary-phase and exponential-phase cultures to which the amoeba-grown *Legionella* cells were compared.

Identification of enhanced resistance to stress (Ers treatment). Intracellular multiplication of *L. pneumophila* in the amoeba *Acanthamoeba polyphagia* enhances resistance to antibiotic and biocide stresses (9, 10). In the previous studies, amoeba-grown bacteria were isolated from the medium 3 days after infection. *Legionella* replicates intracellularly, lyses the amoeba host, and begins to be released into the infection medium within a few hours after infection (5, 36, 71). Thus, the amoeba-grown bacteria used for antibiotic and biocide studies were exposed for as long as 3 days to the infection medium, a neutral-pH phosphate-saline buffer that does not support *L. pneumophila* replication. It is possible that incubation in the infection medium—without prior exposure to amoebae—contributes to phenotypic changes attributed to intra-amoebic growth. In our experiments (Fig. 1) and in the experiments with *H. vermiformis* (2), intra-amoebic *L. pneumophila* cells were recovered by shear or osmotic lysis of the amoebae, and any bacteria released into the infection medium by bacterial lysis of amoebae were removed and not used for stress studies.

We tested if resistance to acid and H_2O_2 stresses is enhanced by incubation of *Legionella* in the infection medium for *A. castellanii*, AC buffer (5, 19, 20, 50, 71), a phosphate-saline buffer similar to *A. polyphagia* infection medium (2). Incubation in AC buffer in the absence of amoebae significantly increased the resistance of stationary-phase *L. pneumophila* cultures to pH 3 and H_2O_2 (Fig. 1). We called this incubation in AC buffer Ers treatment (enhanced resistance to stress) to indicate its effect on resistance of stationary-phase cultures to acid and H_2O_2.

Ers treatment enhances stress resistance of *Legionella katA* and *icm*/*dot* mutants. Mutants with defective virulence phenotypes were not examined in prior studies of the effect of in vitro and intra-amoebic growth on stress resistance (2, 9, 10). Therefore, we examined the effects of these growth conditions on an *L. pneumophila* periplasmic catalase-peroxidase mutant with a Gm^r element inserted in the *katA* gene. This mutant is defective in intracellular multiplication in primary macrophages, macrophage lines, and *A. castellanii* (5, 6). Stationary-phase cultures of the *katA* oxidative stress response mutant were considerably more sensitive than wild-type stationary cultures to pH 3 and H_2O_2 stresses (compare Fig. 2 with Fig. 1). However, Ers treatment restored the level of resistance of the *katA* mutant to that of wild-type stationary-phase cultures for both acid and H_2O_2 stresses (compare Fig. 2 with Fig. 1).

Next, the effect of growth conditions on acid resistance was examined in *icm*/*dot* mutants defective in intracellular multiplication in HL60-derived macrophages and *A. castellanii* (*icmF*, *icmP*, and *icmS* Tn insertion mutants) (71). (Fig. 3) Ers treatment increased the level of resistance to that of Ers-treated wild-type *Legionella* for the *icmF* and *icmP* mutants, while the *icmS* mutant was not responsive to Ers treatment. Restoration of the response to Ers treatment in the complemented *icmS* mutant shows that loss of the response is related to inactivation of *icmS*. These results suggest that *icmS* is essential for the

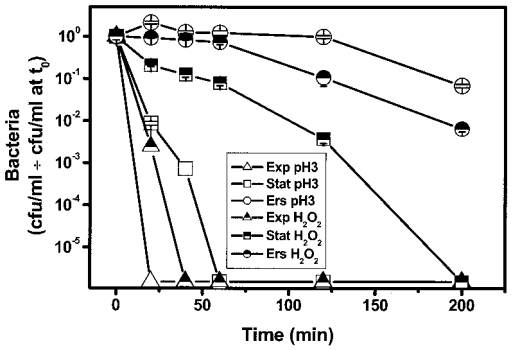

FIG. 2. Effect of growth conditions on the stress resistance of the *katA* null mutant. Exponential-phase (Exp), stationary-phase (Stat), or Ers-treated stationary-phase (Ers) cultures of the *katA*::Gm^r null mutant or bacteria recovered from *A. castellanii* following intracellular multiplication (Acca) were challenged with pH 3 or 1 mM H_2O_2 and titrated after different times (mean ± standard deviation). The points on the *x* axis are the values when there were no CFU.

stress response to Ers treatment but that neither *katA*, *icmF*, nor *icmP* is required. Since for all mutants there was increased resistance of stationary-phase cultures compared to exponential-phase cultures, neither *katA*, *icmF*, *icmP*, nor *icmS* is required for the cross-resistance stress phenotype (Fig. 2 and 3).

Ers treatment suppresses the defective entry of *katA*, *katB*, and *dot*/*icm* mutants into amoebae. The entry of amoeba-grown wild-type *L. pneumophila* into *A. castellanii* is increased 10-fold compared to the entry of stationary-phase bacteria (20). The amoeba-grown bacteria used for this study were isolated from the AC buffer infection medium following lysis of amoeba hosts by *Legionella*. Since both intra-amoebic growth and Ers treatment enhanced the acid and H_2O_2 resistance of wild-type *Legionella* (Fig. 1), we examined if Ers treatment enhanced entry into *A. castellanii*. No significant change in entry was seen following Ers treatment of wild-type *Legionella* (Fig. 4A).

We next examined if Ers treatment enhanced entry of *katA* and *icm* mutants, mutants that exhibited defective intracellular multiplication in *A. castellanii*. Consistent with previous results obtained by using fluorescence microscopy (39), *icmF*, *icmP*, and *icmS* mutants were reduced in entry when stationary-phase cultures of *Legionella* were used to infect *A. castellanii* (Fig. 4A). Defective entry for the *katA* mutant was not examined previously. Remarkably, entry of the *katA*, *icmF*, and *icmP* mutants was increased 10- to 30-fold when Ers-treated stationary-phase cultures were used to infect *A. castellanii* (Fig. 4A). Ers treatment restored entry of the *katA* mutant to wild-type values, and entry of *icmF* and entry of *icmP* were restored to 40% of the wild-type values. These effects parallel the effects of Ers treatment on stress resistance in that the *katA*, *icmF*, and *icmP* mutants (Fig. 2 and 3A and B) were responsive, while the *icmS* mutant was not affected by Ers treatment (Fig. 3C).

When the *icm* mutants were complemented with wild-type alleles on plasmids, entry of stationary-phase cultures was no longer defective compared to entry of wild-type strain JR32 containing the empty complementing vector (Fig. 4B). These findings are consistent with the results of previous studies that demonstrated complementation of the entry defect in stationary-phase cultures (39). Similarly, the entry of Ers-treated complemented mutants was not significantly different from the entry of the wild type containing the empty vector (Fig. 4B). The complementation data demonstrated that suppression of defective entry by Ers treatment was associated with loss of function of the *katA*, *icmF*, and *icmP* genes and was unlikely to be associated with spontaneous mutations outside the inactivated genes.

To ascertain if Ers treatment suppresses defective entry of other *Legionella* mutants, we examined a *katB* null mutant, whose intracellular multiplication phenotype was like that of the *katA* mutant (5, 6), and null mutants with mutation in four other *icm*/*dot* genes. One of these was a *dotA* mutant, with a Tn insertion in the *dotA* gene, required for many aspects of *Legionella* virulence when stationary-phase cultures are used for infection, from entry through cytotoxicity to the host cell (11, 43, 44, 63, 64). All five mutants exhibited statistically significant

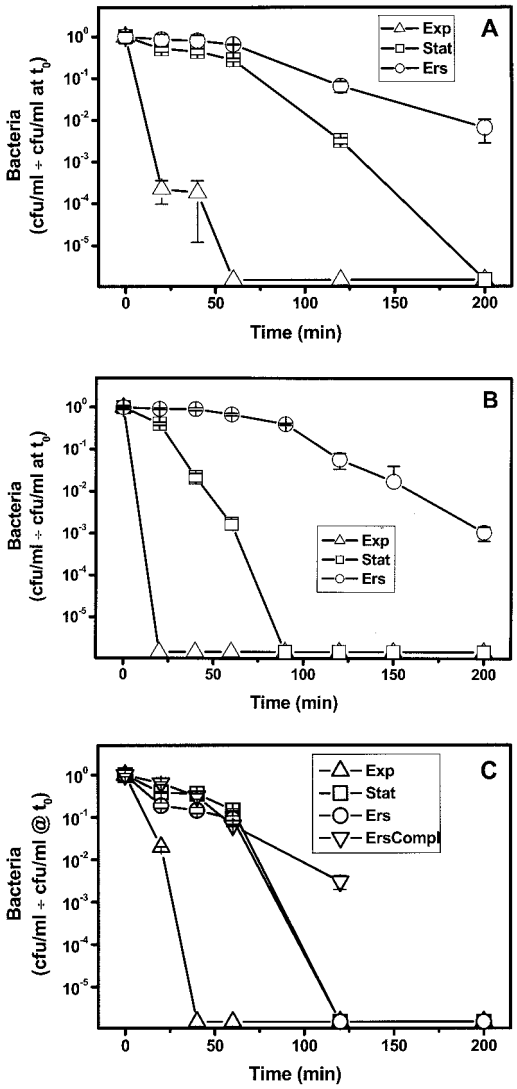

FIG. 3. Effect of growth conditions on acid resistance of *icm* null mutants. Acid challenge experiments were performed with *icmF* (A), *icmP* (B), and *icmS* (C) null mutants. Exponential-phase (Exp), stationary-phase (Stat), or Ers-treated stationary-phase (Ers) cultures were challenged with pH 3 and titrated after different times (mean ± standard deviation). Ers compl, Ers-treated stationary-phase culture of *icmS* mutant containing an *icmS*$^+$ complementing plasmid. The points on the *x* axis are the values when there were no CFU.

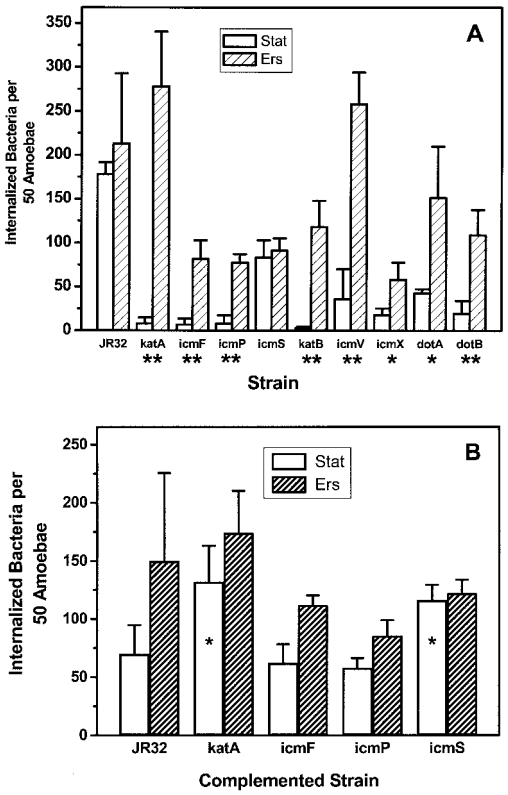

FIG. 4. Ers treatment reverses the defect in entry of *katA*, *katB*, and *icm*/*dot* mutants into *A. castellanii*. *A. castellanii* was infected at an MOI of 3 to 5 with wild-type *L. pneumophila* strain JR32 or a mutant strain grown to the stationary phase (Stat) or after Ers treatment of stationary-phase cultures (Ers). Internalized bacteria were quantified by the fluorescence microscopy method (mean ± standard deviation). (A) Strains containing a GFP-expressing plasmid. (B) Strains containing the pJN105-hygro-GFP plasmid and a second plasmid, pMMB207αB-Km14 for wild-type strain JR32 or a complementing plasmid for mutant strains. *P* values were determined by one-sided *t* tests by comparing entry of stationary-phase and Ers-treated cultures of the same strain (A) or by comparing a mutant stationary-phase or Ers-treated culture with a similar wild-type culture (B) (two asterisks, $P < 0.005$; one asterisk, $P < 0.02$; no asterisk, $P > 0.05$).

increases in entry following Ers treatment, which ranged from 3- to 4-fold for the *icmX* and *dotA* mutants to 40-fold for the *katB* mutant (Fig. 4A). Moreover, Ers treatment restored the levels of entry to >50% of the wild-type value for the *katB*, *icmV*, *dotA*, and *dotB* mutants and to 30% of the wild-type value for the *icmX* mutant.

These data show that the defective entry of *katA*, *katB*, *icmF*, *icmP*, *icmV*, *icmX*, *dotA*, and *dotB* mutants into *A. castellanii* is conditional and dependent on culture conditions. The entry deficit was evident in stationary-phase cultures, as described previously (39), but was suppressed by Ers treatment of stationary-phase cultures. To our knowledge, suppression of an entry defect by varying the culture conditions of a *Legionella icm*/*dot* or other virulence mutant has not been reported previously.

Ers treatment suppresses the defective entry of *katA*, *katB*, and *icm*/*dot* mutants into HL60-derived macrophages. Many *L. pneumophila icm*/*dot* mutants are defective in entry (39) and in intracellular multiplication (71) in both macrophages and amoeba hosts. We therefore examined if Ers treatment, which suppressed the defective entry of stationary-phase cultures of *katA*/*B* and *icm*/*dot* mutants into amoebae, similarly suppressed the defective entry into HL60-derived macrophages. Consistent with previous work (39), stationary-phase cultures of *icm*/*dot* mutants exhibited decreased entry into HL60-derived macrophages (Fig. 5). Ers treatment did indeed suppress the defective entry of *katA*, *katB*, *icmF*, *icmV*, *icmX*, *dotA*, and *dotB* null mutants into macrophages. Statistically significant two- to ninefold increases were seen, and for all mutants entry into macrophages was restored to wild-type levels. Entry of the *icmP* mutant into macrophages was not significantly changed, and entry of the *icmS* mutant was decreased.

Intra-amoebic growth suppresses the defective entry of the *katA* virulence mutant into HL60-derived macrophages. Only wild-type *L. pneumophila* cells were used in previous studies that demonstrated enhanced entry into macrophage and amoeba hosts following intra-amoebic growth (19, 20). To examine the effect of intra-amoebic growth on a *Legionella* mutant with defective intracellular multiplication, we used the *katA* mutant, which replicates in *A. castellanii* at a reduced rate (5) and was defective in amoeba and macrophage entry (Fig. 4A and 5). The *dotA* mutant and most other *icm*/*dot* Tn insertion mutants were unsuitable for this experiment because they are nonreplicative in *A. castellanii* (71). This is likely why only wild-type *L. pneumophila* was examined in previous studies of phenotypic changes following intra-amoebic growth (9, 10, 19, 20, 32).

A. castellanii was infected with wild-type or *katA Legionella*, and intracellular bacteria were isolated 2 days later by shear lysis of the amoeba host, as was done for isolation of amoeba-grown *Legionella* for the acid and H_2O_2 stress experiments (Fig. 1). Entry into HL60-derived macrophages was quantified by examining the gentamicin protection, the method used previously to show enhanced entry of amoeba-grown wild-type *Legionella* (19, 20). The 8.1-fold increased entry of wild-type *L. pneumophila* which we found is in good agreement with the previously described 10-fold increased entry of amoeba-grown wild type into cultured macrophage lines (20). Entry of the *katA* mutant was increased 12-fold, in good agreement with the 9.3-fold increase determined by GFP fluorescence after Ers

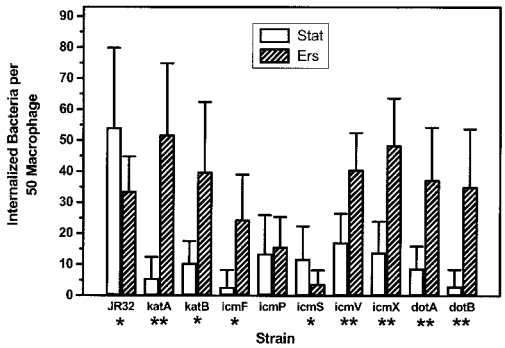

FIG. 5. Ers treatment reverses the defect in entry of *katA*, *katB*, and *icm*/*dot* mutants into HL60-derived macrophages. The entry of wild-type strain JR32 or a mutant strain into macrophages derived from HL60 monocytes (MOI, 100) was determined as described for entry into *A. castellanii* in the legend of Fig. 4. *P* values were determined by one-sided *t* tests by comparing entry of stationary-phase (Stat) and Ers-treated (Ers) cultures of the same strain (one asterisk, $P < 0.05$; two asterisks, $P < 0.005$; no asterisk, $P > 0.05$).

TABLE 2. Effect of Ers treatment on the antibiotic resistance of stationary-phase *Legionella*[a]

Antibiotic	Concn (μg/ml)	JR32			*dotA* mutant		
		% Survival		Ers/Stat	% Survival		Ers/Stat
		Stat	Ers		Stat	Ers	
Gentamicin	100	$1.75 \times 10^{-3} \pm 1.2 \times 10^{-3}$	0.73 ± 0.41	420	0.041 ± 0.0042[b]	0.87 ± 0.06	21
Erythromycin	100	19 ± 2	46 ± 16	2.4	2.5 ± 0.9	12 ± 6	4.8
Rifampin	100	54 ± 0.5	69 ± 16	1.3	0.74 ± 0.04	70 ± 11	95

[a] Bacteria in the stationary phase (Stat) or following Ers treatment after the stationary phase (Ers) were resuspended in phosphate-buffered saline at a concentration of 1×10^8 to 5×10^9 CFU/ml, incubated at 37°C for 40 min with the concentration of antibiotic indicated, and then titrated on CYE plates. The titer obtained compared to titer before antibiotic treatment is expressed as the percent survival; the values are means ± standard deviations.

[b] The higher level of survival of the stationary-phase *dotA* mutant than of stationary-phase strain JR32 is attributable to the kanamycin resistance element in *dotA*::Tn*903*, which conveys a low level of gentamicin resistance.

treatment of this mutant (Fig. 5). Thus, intra-amoebic growth, like Ers treatment, can suppress the defective entry of the *Legionella katA* null mutant.

Ers treatment increases antibiotic resistance of wild-type and *dotA* mutant *L. pneumophila*. Increased resistance to biocides used in water purification and to antibiotics used to treat *Legionella* infections, including erythromycin (65), is shown by *Legionella* grown in the amoeba *A. polyphagia* (9, 10). Since both intra-amoebic growth and Ers treatment increased resistance to acid and H_2O_2 stresses (Fig. 1) and suppressed defective entry of the *katA* mutant, we examined if Ers treatment increased antibiotic resistance.

The resistance of wild-type *Legionella* to gentamicin and erythromycin was increased 400- and 2-fold, respectively, following Ers treatment of stationary-phase cultures (Table 2). The resistance of the *dotA* Tn insertion mutant to gentamicin, erythromycin, and rifampin was increased 20-, 5-, and 95-fold, respectively, by Ers treatment. Previous studies reported that intra-amoebic growth increased resistance to erythromycin and rifampin 100- and 1,000-fold, respectively, for wild-type *Legionella* (10), but *icm*/*dot* mutants were not examined. In the previous studies the workers used 10- to 20-fold-lower drug concentrations and resuspended *Legionella* in fresh broth during the 6 to 24 h of antibiotic challenge. In our experiments stationary-phase or Ers-treated cultures were exposed to antibiotics for 40 min in AC buffer, which does not support *Legionella* growth. Thus, the Ers-induced increase in antibiotic resistance is qualitatively similar to that observed for intra-amoebically grown *Legionella* (10), but quantitative comparisons are not possible due to differences in experimental design.

In the studies that demonstrated increased antibiotic resistance following intra-amoebic growth, *L. pneumophila* cells were isolated from the infection medium 3 days after infection of *A. polyphagia* (10). As noted above, *Legionella* begans to be released into the medium within a few hours after infection of amoebae. The 16- to 19-h duration of Ers treatment, in which *Legionella* was exposed to a phosphate-saline buffer similar to the *A. polyphagia* infection medium (10), is within the 3-day time frame of exposure to infection medium in the *A. polyphagia* studies. Therefore, exposure to the infection medium following *Legionella* lysis of amoeba hosts may have contributed to the increased antibiotic resistance previously attributed to intra-amoebic growth (10).

Mechanistic aspects of Ers suppression of mutant phenotypes in *katA*, *katB*, and *icm*/*dot Legionella* mutants Ers treatment suppressed defective entry associated with null mutations in a diverse group of genes encoding structural and chaperone components of the Icm/Dot type IVB secretion apparatus (22, 63, 69, 74, 80) and catalase-peroxidase enzymes. To investigate the mechanism(s) by which suppression was accomplished, we first considered the possibility that Ers treatment induces multiple *icm*/*dot* genes, which then compensate for inactivation of individual *icm*/*dot* genes. This mechanism is unlikely because several *icm*::*lacZ* translational fusions show no significant change in expression during incubation of stationary-phase cultures in AC buffer (Gil Segal, Tel Aviv University, 2004, personal communication). In addition, induction of *icm*/*dot* genes is an unlikely mechanism for suppression of entry defects in mutants with mutations in KatA and KatB catalase-peroxidase enzymes that are not components of the Icm/Dot type IVB secretion machinery. Second, we considered the possibility that Ers treatment enhances adhesion of *Legionella* to host cells, thus enhancing entry. This possibility is not supported by the results obtained by the fluorescence microscopy method that distinguished internalized bacteria from external bacteria. Specifically, Ers treatment does not increase association of external *L. pneumophila* with amoebae or macrophage envelopes. Third, we considered the possibility that Ers treatment increases piliation, which has recently been implicated in entry by isolation of a gene with homology to the genes encoding pilin subunits of type IV secretion systems (60). This possibility is not supported by the results of electron microscopy with phosphotungstate and ammonium molybdate negative staining. No difference in piliation was seen between stationary-phase and Ers-treated cultures of the *dotA* mutant, which responded to Ers treatment, or the *icmS* mutant, which did not respond to Ers treatment (data not shown). Fourth, we considered the possibility that Ers treatment changes the bacterial surface morphology, eliciting changes responsible for enhanced entry. This possibility is not supported by electron microscopy data for stationary-phase and Ers-treated cultures of *dotA* and *icmS* mutants. In both mutants and in both culture conditions, the cell envelope is smooth and unruffled, and the speckling patterns that are reflective of involutions in the cell surface are comparable. The cell widths are identical for the two mutants and are not changed by the culture conditions, and the cell length is shorter in Ers-treated cultures, which is consistent with starvation, utilization of energy stores, and decreased entry into a replicative state (data not shown). The dramatic differences in cell morphology seen in amoeba-grown

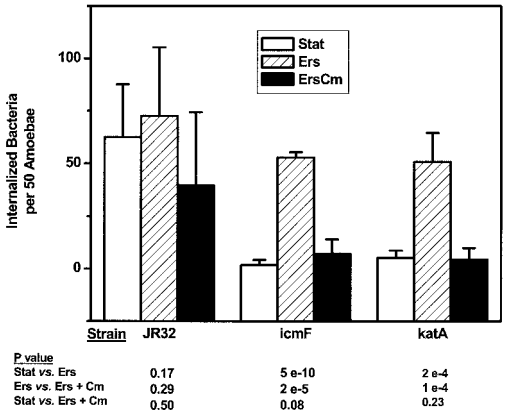

FIG. 6. Chloramphenicol inhibits Ers-enhanced entry into *A. castellanii* for *icmF* and *katA* mutants. Entry into *A. castellanii* was determined for wild-type strain JR32 and *icmF* and *katA* mutant strains as described in the legend to Fig. 4 with stationary-phase cultures (Stat), Ers-treated stationary-phase cultures (Ers), and Ers-treated stationary-phase cultures in the presence of chloramphenicol (ErsCm and Ers + Cm).

Legionella by light and electron microscopy (20, 32) are not evident in Ers-treated cultures. Fifth, we considered the possibility that Ers treatment decreases viability and that enhanced entry can be attributed to increased phagocytosis of dead bacteria. This possibility is not consistent with the viability measurements. The levels of viability of broth-grown stationary-phase cultures of wild-type strain JR32 and the *katA* and *dotA* mutants are 95% ± 5%, 92% ± 3%, and 96% ± 4%, respectively. The levels of viability of Ers-treated broth cultures are 94% ± 5%, 95% ± 2%, and 94% ± 2%, respectively. Thus, Ers treatment does not significantly decrease the viability of broth-grown stationary-phase cultures, and high levels of viability are evident in both stationary-phase and Ers-treated cultures.

Protein expression differences between stationary-phase *L. pneumophila* and intra-amoebically grown bacteria are evident as determined by SDS-polyacrylamide gel electrophoresis (20). To establish if protein expression is required for Ers suppression of defective entry, entry into *A. castellanii* was determined for stationary-phase cultures and for stationary-phase cultures exposed to Ers treatment in the absence or in the presence chloramphenicol (Fig. 6). Chloramphenicol inhibition of protein synthesis abolished Ers treatment-enhanced entry of *katA* and *icmF* mutants. Entry of wild-type *Legionella* into amoebae was not significantly altered by Ers treatment in the absence or presence of chloramphenicol. These results demonstrated that protein synthesis occurs during Ers treatment and is required for Ers suppression of the entry defect in *katA* and *icmF* mutants and presumably is required for suppression of other mutants.

Proteomic analysis of Ers treatment-induced changes in expression. To directly examine expression changes implicated in the chloramphenicol inhibition experiments, stationary-phase and Ers-treated *L. pneumophila* cultures were compared by using two-dimensional gel electrophoresis, MALDI-TOF analysis of tryptic digests, and data mining of the *L. pneumophila* genome. This approach has been used with other bacterial species to assess changes in protein expression in different culture conditions (12, 34, 56, 82). Two-dimensional gels were prepared by using proteins in sonic extracts of *katA*, *dotA*, *dotB*, *icmF*, *icmS*, and *icmP* mutant strains.

Intensity differences between stationary-phase and Ers-treated cultures were consistently seen in about 15 spots. One protein whose level was consistently decreased after Ers treatment is the *Legionella* homologue of cell division protein FtsZ, ORF lpg 2609 in the *L. pneumophila* genome (James Russo, Columbia Genome Center, personal communication). A decrease in the FtsZ level is consistent with the decreased replication expected in cultures exposed to Ers treatment, in which stationary-phase bacteria are incubated in a nutrient-deficient buffer. The decreased expression of FtsZ after Ers treatment is consistent with electron microscopy changes in cell length that are suggestive of decreased replication after Ers treatment. All strains whose defective entry into macrophages was suppressed by Ers treatment (*katA*, *dotA*, *dotB*, and *icmF* mutants) (Fig. 5) showed a large increase in the intensity of a spot in Ers-treated cultures compared to stationary-phase cultures (Fig. 7). In strains whose defective entry into macrophages was not suppressed by Ers treatment (*icmP* and *icmS* mutants) (Fig. 5), the

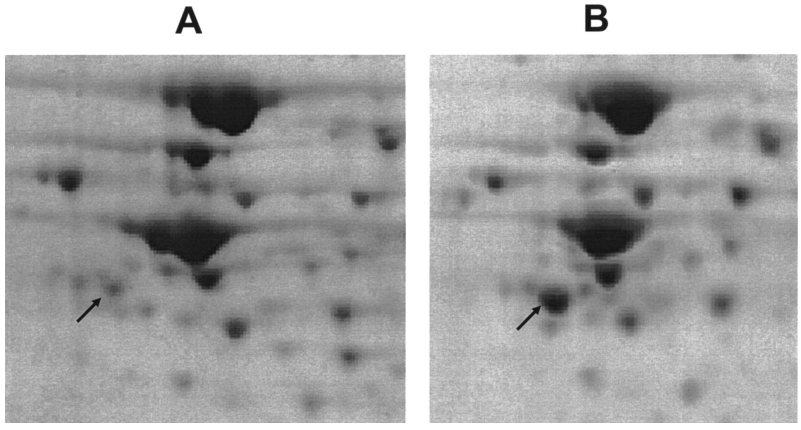

FIG. 7. Two-dimensional gel electrophoresis analysis of *L. pneumophila katA* mutant. Isoelectric focusing (horizontal) and SDS-polyacrylamide gel electrophoresis (vertical) were performed with sonic extracts from stationary-phase (A) and Ers-treated stationary-phase (B) cultures. The regions shown are ~20% of each gel surrounding ORF lpg2222 (arrows) (calculated molecular weight, 41,400; calculated pI 5.35). The amount of protein loaded in panel A was greater than the amount loaded in panel B.

intensity of the spot was not increased by Ers treatment. The protein in this spot, ORF lpg2222 in the *L. pneumophila* genome (Russo, personal communication), is predicted (49; http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) to contain eight consecutive tetratricopeptide repeats (TPRs) (13) and is highly homologous (E value, $<e^{-25}$) to known TPR proteins. The TPR motif is a degenerate repeat consisting of ~34 amino acids that functions in protein-protein interactions, often in multiprotein complexes. This motif is found in yeast transcription factors, eukaryotic chaperones involved in protein import to peroxisomes, and mitochondria (13) and has recently been implicated in chaperones and regulators of bacterial type III secretion systems (57).

DISCUSSION

Environmental stress plays a major role in the transition of *L. pneumophila* from a planktonic pond resident to an intracellular pathogen of alveolar macrophages. In the laboratory, growth to the stationary phase is widely used to model this stress-induced transition. When stationary-phase cultures are used as the infectious inocula, *Legionella* homologues of enteric regulators of stress, starvation, and/or stationary-phase responses play a role in expression of intracellular multiplication and other *Legionella* virulence phenotypes. Among these regulators are *L. pneumophila* homologues of RpoS (4, 36, 84), an alternative sigma factor, GacA (LetA) (31, 37), a global activator, and CsrA (28, 51), an RNA-binding protein in the carbon starvation response.

A major focus in studying the pathways connecting stress with virulence phenotypes is the *icm*/*dot* loci, which encode a type IVB secretion apparatus proposed to translocate *Legionella* proteins into host cells. The type IVB apparatus and its translocated proteins are involved in entry, evasion or delay of fusion between lysosomes and *Legionella*-containing phagosomes, association of the phagosome with the endoplasmic reticulum, intracellular multiplication, cytotoxicity, and the release of replicated bacteria from host cells (63, 69, 73, 80). However, *Legionella* RpoS and GacA have no significant effect on the expression of the *icm*/*dot* transcriptional units (31, 84). CpxR, a regulator of the enteric response to envelope stress (58), activates expression of IcmR (30), which influences formation of pores or channels in host cells by IcmQ (26). The effect of CsrA on *icm*/*dot* gene expression has not been reported. Since these stress regulators have a limited effect or no effect on expression of the *icm*/*dot* genes, they appear to control non-*icm*/*dot* virulence genes outside the *icm*/*dot* loci.

The presence of *icm*/*dot*-independent virulence genes is indicated by data showing that certain attributes of the *Legionella*-containing phagosome do not require a functional Icm/Dot apparatus. DotA, an essential component of the Icm/Dot secretion apparatus (11, 43, 44, 63, 64), and DotB (72) are not required for the delayed acquisition of cathepsin D or Texas Red ovalbumin lysosomal markers by *Legionella*-containing phagosomes (43). These findings indicate that *icm*/*dot*-independent genes are present. Icm/Dot-independent genes are non-*icm*/*dot* genes that play a role in *Legionella* virulence phenotypes in the absence of fully functional *icm*/*dot* genes. The identities of such *icm*/*dot*-independent genes and methods to screen for them are poorly defined.

In this paper we propose Ers treatment as an avenue for identifying *icm*/*dot*-independent *Legionella* virulence factors. In Ers treatment, broth-grown stationary-phase cultures are incubated overnight in AC buffer, a pH 6.5 phosphate-saline buffer which does not support replication of *L. pneumophila* (39, 50, 71). When stationary-phase cultures of *icm*/*dot* mutants are used for infection, defective entry into amoeba and macrophage hosts is observed (39). When the same *icm*/*dot* mutants are subjected to Ers treatment, entry into both amoeba and macrophage hosts is significantly increased. Entry is restored to wild-type or nearly wild-type values for *dotA*, *dotB*, *icmF*, *icmV*, and *icmX* Tn insertion mutants. Ers treatment also significantly increases and restores to wild-type values the entry of null mutants with mutations in the *katA*- and *katB*-encoded catalase-peroxidase enzymes, which are similarly defective in entry when stationary-phase cultures are used for infection. To our knowledge, Ers treatment is the first instance of suppressing a virulence defect in an *L. pneumophila* mutant by a change in culture conditions. For a null mutant with a mutation in the *Legionella* general stress gene, *gspA*, intra-amoebic growth suppresses sensitivity to oxidative, osmotic, acid, and thermal stresses (2). However, the *gspA* mutant is not defective in intracellular multiplication and cytotoxicity.

Ers suppression of defective entry indicates that Ers treatment-induced changes in gene expression can restore entry into amoeba and macrophage hosts to wild-type values in the absence of the *icm*/*dot* or the catalase-peroxidase gene that is inactivated. Therefore, Ers treatment is a screening procedure for *icm*/*dot*-independent factors that act in *Legionella* entry into eukaryotic hosts, an early step in *Legionella* pathogenicity. A remarkable feature of Ers treatment is its pleiotropic nature. Ers treatment suppresses defective entry into both amoebae and HL60-derived macrophages. It restores entry for mutants with mutations in catalase-peroxidase enzymes (periplasmic KatA and cytosolic KatB) (6) and membrane-associated (DotA, IcmV), cytosolic (DotB), and predicted periplasmic (IcmX) components of the type IVB secretion apparatus (69). Thus, identifying the molecular players in Ers treatment suppression and their mechanism of action may lead to new insights into *L. pneumophila* pathogenicity.

Since protein synthesis is required for Ers suppression, analysis of expression differences between stationary-phase and Ers-treated cultures is a means of investigating the mechanism of Ers suppression and identifying *icm*/*dot*-independent virulence factors. We developed a proteomic approach for identifying Ers-induced expression changes and observed Ers-dependent induction of ORF lpg2222 in the *L. pneumophila* genome. The ORF lpg2222 protein is predicted to contain TPRs. This protein-protein interaction motif is also present in EnhC (ORF lpg2639), previously associated with enhanced entry in a screening of mutagenized *L. pneumophila* strain AA100 for enhanced entry into macrophage and epithelial cell hosts (21). Enhanced entry of the *enhC* mutant is attributed to elevated *enhC* expression. A comparison of *enhC* mutant and wild-type stationary-phase cultures revealed no difference in pili or flagella, similar to the results obtained when we compared stationary-phase and Ers-treated *L. pneumophila*. It is not known if *enhC* is upregulated by Ers treatment, if elevated expression of *enhC* suppresses the defective entry of *dotA*, *dotB*, and other

icm/*dot* mutants, or if expression of *enhC* or ORF lpg2222 is influenced by *Legionella* stress regulators.

Association of two TPR-containing proteins with entry into macrophage hosts raises the possibility that the TPR motif is important for protein-protein interactions in this step in *Legionella* infection. EnhC contains 13 TPR motif regions (49; http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). ORF lpg 2222 contains eight TPR motifs, one 35 residues long and seven 36 residues long. Initially found in yeast cell division cycle proteins, TPR motifs participate in protein-protein interactions of transcription factors and cochaperones and in the import of proteins into the matrix of peroxisomes and mitochondria (13). Recently, TPR motifs have been predicted in known chaperones and regulators of the bacterial type III secretion apparatus (57). However, no TPRs are predicted (49; http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) in VirE1 and IcmR, which are known chaperones in the type IVB secretion systems of *Agrobacterium tumefaciens* (24) and *L. pneumophila* (22, 25). Knowledge of the protein partners with which the TPR motif proteins EnhC and ORF lpg2222 interact—whether they interact with Icm/Dot proteins, with one another, or with novel proteins—is important information for determining their roles in the entry of *L. pneumophila* into host cells. For ORF lpg2222, this information may also provide insight into the *icm*/*dot*-independent factors that act in suppression of defective entry of *icm*/*dot* and *katA*/*B Legionella* mutants into amoeba and macrophage hosts by Ers treatment.

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