Integrative Conjugative Element ICE-βox Confers Oxidative Stress Resistance to *Legionella pneumophila In Vitro* and in Macrophages

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ABSTRACT **Integrative conjugative elements (ICEs) are mobile blocks of DNA that can contribute to bacterial evolution by self-directed transmission of advantageous traits. Here, we analyze the activity of a putative 65-kb ICE harbored by *Legionella pneumophila* using molecular genetics, conjugation assays, a phenotype microarray screen, and macrophage infections. The element transferred to a naive *L. pneumophila* strain, integrated site-specifically, and conferred increased resistance to oxacillin, penicillin, hydrogen peroxide, and bleach. Furthermore, the element increased survival of *L. pneumophila* within restrictive mouse macrophages. In particular, this ICE protects *L. pneumophila* from phagocyte oxidase activity, since mutation of the macrophage NADPH oxidase eliminated the fitness difference between strains that carried and those that lacked the mobile element. Renamed ICE-βox (for β-lactam antibiotics and oxidative stress), this transposable element is predicted to contribute to the emergence of *L. pneumophila* strains that are more fit in natural and engineered water systems and in macrophages.**

IMPORTANCE **Bacteria evolve rapidly by acquiring new traits via horizontal gene transfer. Integrative conjugative elements (ICEs) are mobile blocks of DNA that encode the machinery necessary to spread among bacterial populations. ICEs transfer antibiotic resistance and other bacterial survival factors as cargo genes carried within the element. Here, we show that *Legionella pneumophila*, the causative agent of Legionnaires' disease, carries ICE-βox, which enhances the resistance of this opportunistic pathogen to bleach and β-lactam antibiotics. Moreover, *L. pneumophila* strains encoding ICE-βox are more resistant to macrophages that carry phagocyte oxidase. Accordingly, ICE-βox is predicted to increase the fitness of *L. pneumophila* in natural and engineered waters and in humans. To our knowledge, this is the first description of an ICE that confers oxidative stress resistance to a nosocomial pathogen.**

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Bacteriophage and transposable elements speed the spread of advantageous traits among bacterial populations, promoting genome diversity and evolution. The nosocomial lung pathogen *Legionella pneumophila* exhibits an extraordinary amount of genome plasticity, as up to 30% of six sequenced strain genomes is unique (1). Much of this variance is attributed to the acquisition of mobile elements (1), including integrative conjugative elements (ICEs). ICEs are a class of transposons that encode type IV secretion systems (T4SS) that transfer by bacterial conjugation the core element as well as cargo genes that may confer fitness traits to the host (2). ICEs efficiently induce their own excision from the bacterial chromosome and subsequent site-specific integration into the chromosome of a new bacterium, ensuring the elements' propagation by the host replication machinery. By this mechanism, pathogens have acquired a variety of traits, including antibiotic resistance, biofilm formation, metal ion resistance, and host invasion factors (2).

A number of ICEs have been identified in different strains of *L. pneumophila*. *L. pneumophila* strain Corby carries mobile ICEs (3, 4). The heterogeneity of the *L. pneumophila* genome was probed in a hybridization study of 217 clinical and environmental isolates (5). Three of the regions that are highly variable between isolates resemble ICEs that are predicted to enhance versatility. The *lvh* element of *L. pneumophila* strain Philadelphia-1, which is carried by 67% of isolates examined, restores entry and intracellular multiplication defects to mutants deficient in the canonical *dot/icm* T4SS (6, 7). A second, highly variable genomic region less frequent in this collection of *L. pneumophila* isolates (18%) is LpPI-1 (8). This 65-kb element (here renamed ICE-βox) is predicted to encode machinery for excision and transfer as well as a number of putative virulence factors and detoxifying enzymes (8).

To investigate the mobility and contribution of ICE-βox to *L. pneumophila* fitness, we applied genetic assays, a Biolog phenotype microarray, *in vitro* growth analysis, and macrophage infection studies on strains that contain or lack the element.

RESULTS

***L. pneumophila* ICE-βox excision, transfer, and site-specific integration.** ICEs promote their own conjugative transfer by encoding a type IV secretion system. To determine whether ICE-βox can spread to a naive bacterial recipient, we performed conjugation assays using a donor that carried a genetically marked ICE-βox. For this experiment, we exploited known differences in two derivatives of the *L. pneumophila* strain Philadelphia-1. Strain Lp02

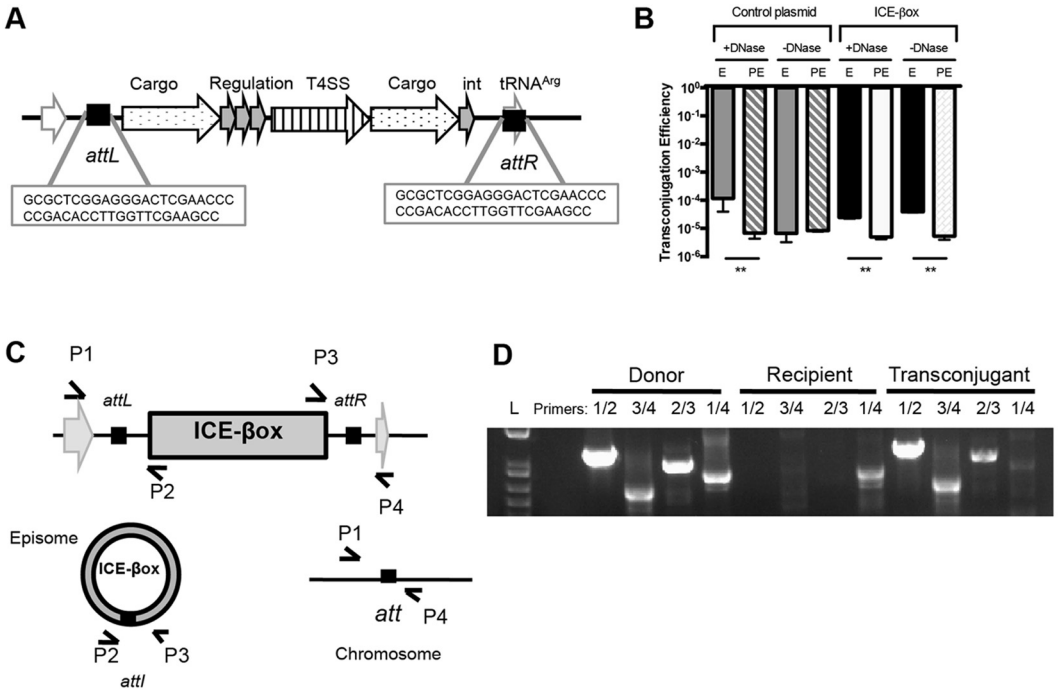

FIG 1 ICE-βox is a mobile genetic element. (A) Schematic of ICE-βox. The 65-kb locus is predicted to harbor 38 cargo (spotted arrows), 4 regulatory (gray arrows), and 18 type IV secretion system (T4SS; striped arrow) genes. ICE-βox is flanked by 43-bp direct nucleotide repeat regions deduced to serve as attachment sites (*attL* and attR). The drawing is not to scale. (B) ICE-βox transfer by exponential- and post-exponential-phase cells is resistant to DNase I. Plating on selective medium identified control plasmid or ICE-βox transconjugants. The mean efficiency ± SEM was calculated from three experiments as the number of ICE-βox-positive JR32 recipient cells per donor cell. More efficient conjugation by E-than by PE-phase cells was statistically significant according to the Student *t* test (**, $P < 0.01$). (C) Schematic of ICE-βox excision assay. Specific primer sets P1/P2 and P3/P4 amplify the *attL* and attR junction fragments, respectively, whereas P2/P3 generate a product (attI) from excised ICE-βox and P1/P4 amplify the attachment site remaining in the chromosome after excision (att). (D) PCR detects circularized ICE-βox. Using the PCR assay described in panel A and genomic DNA isolated from *L. pneumophila* donor (Lp02, MB1353), recipient (JR32, MB1354), and transconjugant (JR32 + ICE-βox, MB1354), junction fragments for integrated (*attL* and attR) and excised-ICE (attI) forms of ICE-βox were detected as well as the site remaining after excision (att).

carries ICE-βox, but JR32 does not (9); therefore, ICE-βox-marked Lp02 cells served as the donor and naive JR32 cells served as the recipients. Indeed, like a control plasmid that carries an *oriT* sequence, ICE-βox transferred from donor to recipient cells by a process insensitive to exogenous DNase I (Fig. 1B). ICE-βox transfer was ~10-fold more efficient when donor cells were in exponential phase ($P < 0.01$).

To determine whether ICE-βox integrated into the chromosome of the transconjugant strain, we applied a PCR assay (10). Specific primer set (Fig. 1C) P1/P2 or P3/P4 amplifies 5′ or 3′ junction fragments of ICE-βox that are integrated into the chromosome (*attL* and *attR*, respectively), whereas primers P2/P3 generate a product from elements that are excised and circularized (*attI*) and primers P1/P4 amplify the scar site after excision (*att*). Whereas no integrated or excised ICE-βox product was generated from recipient JR32 DNA, both integrated and episomal forms of ICE-βox were detected within a population of cells of the *L. pneumophila* Lp02 donor and the transconjugant strain, and all strains harbored the *att* site necessary for reintegration of ICE-βox into the chromosome (Fig. 1D). In multiple independent experiments, we consistently observed less *attI* and *att* product in the transconjugant population. Perhaps, variability in band intensity for the *attI* and *att* forms of ICE-βox in the transconjugant reflects differential regulation of excision in this strain background.

ICEs integrate into bacterial chromosomes by homologous recombination of direct nucleotide repeat sequences (2). To confirm the integration site of the element, the DNA sequence of the chromosomal and episomal amplicons (*attL*, *attR*, and *attI*) generated as described for Fig. 1D was determined. An identical 43-bp sequence flanked each side of ICE-βox and the excised form (Fig. 1A). One copy of the repeat sequence was located within a chromosomal $tRNA^{Arg}$ gene, consistent with other known ICEs and the genomic sequences of the Philadelphia-1, Lp02, and JR32 strains (9, 11).

Since the *lvh* ICE excises more efficiently in post-exponential-phase cells (see Fig. S1A in the supplemental material) (10), we measured the impact of growth phase on ICE-βox excision. Unlike *lvh*, ICE-βox excision is not sensitive to growth phase in rich medium. Analysis by quantitative PCR (qPCR) of template DNA isolated from cultures at different growth phases determined an excision ratio of ~3×10^{-8} to 5×10^{-8} episomes per chromosome at each time point (see Fig. S1B). Since ICE-βox excises, mobilizes, and integrates site-specifically into a new host chromosome, its designation as an ICE is confirmed.

ICE-βox confers resistance to β-lactam antibiotics, hydrogen peroxide, and bleach *in vitro*. As a strategy to identify traits conferred by ICE-βox, we used Biolog phenotype microarrays to compare the growth of donor and ICE-βox transconjugant strains to that of naive recipient cells. All strains replicated with equal efficiencies in standard growth medium (see Fig. S2A in the sup-

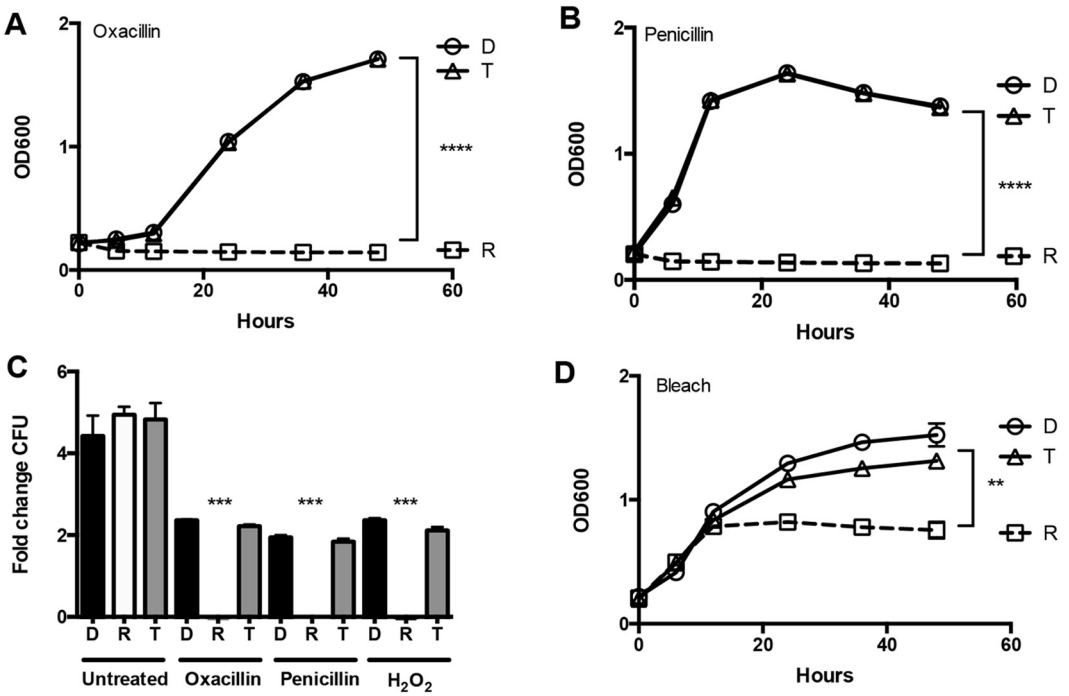

FIG 2 ICE-βox promotes oxidative stress survival. (A and B) ICE-βox confers resistance to oxacillin and penicillin. E-phase cultures of ICE-βox donor (D, circles), recipient (R, squares), or transconjugant (T, triangles) were exposed to 100 μg/ml oxacillin (A) or 2 μg/ml penicillin G (B), and at the times shown, their growth was quantified as optical density at 600 nm by a Bioscreen growth curve analyzer. Shown are means ± SEMs calculated from triplicate samples in one experiment representative of three others. *t* tests indicate that differences between strains that carry or lack ICE-βox are statistically significant (****, $P < 0.001$). (C) ICE-βox increases *L. pneumophila* resistance to oxacillin, penicillin, and hydrogen peroxide. E-phase cultures of ICE-βox donor (D, black bars), recipient (R, white bars), or transconjugant (T, gray bars) strains that contained or lacked ICE-βox were exposed for 6 h to 100 μg/ml oxacillin, 2 μg/ml penicillin G, or 2 mM H_2O_2, and then mean survival ± SEM was calculated from three independent experiments as (CFU treated)/(CFU untreated). *t* tests indicate that differences between strains that carry or lack ICE-βox are statistically significant (***, $P < 0.005$). (D) ICE-βox increases *L. pneumophila* resistance to bleach. E-phase cells of ICE-βox donor (D, circles), recipient (R, squares), or transconjugant (T, triangles) strains were cultured with 0.5 ppm bleach, and optical density at 600 nm was recorded at the times shown using a Bioscreen growth curve analyzer. Shown are means ± SEMs calculated from three replicates in one experiment representative of three others. Multiple *t* tests indicate that differences between strains that carry or lack ICE-βox are statistically significant (**, $P < 0.01$).

plemental material). Of the 200 different carbon sources and growth inhibitors tested, the most striking differences were observed for two β-lactam antibiotics, oxacillin and penicillin. Subsequent growth curve experiments confirmed that the ICE-βox-containing donor and transconjugant *L. pneumophila* strains tolerated these antibiotics but that the naive recipient strain did not (Fig. 2A and B). We verified that ICE-βox promotes bacterial survival and replication by quantifying CFU after a 6-h exposure to 100 μg/ml oxacillin or 2 μg/ml penicillin G (Fig. 2C).

β-Lactam antibiotics are known to induce production of reactive oxygen species (ROS) as an indirect consequence of cross-linking cell wall peptidoglycan (12). Therefore, to test whether ICE-βox confers protection from ROS, we quantified resistance to hydrogen peroxide. Similarly to the β-lactam antibiotics, ICE-βox enhanced resistance to this oxidative stress (Fig. 2C). We next tested the ability of ICE-βox to protect *L. pneumophila* from bleach (NaOCl), a common disinfectant that contains both reactive oxygen and chlorine. As observed for the other oxidative stresses tested, donor and transconjugant strains that carried ICE-βox survived and replicated in culture media supplemented with bleach to 0.5 ppm, a concentration typical of drinking water (Fig. 2D) (13). For all phenotypic tests, three additional independent transconjugant strains and two markerless ICE-βox derivatives exhibited similar patterns of resistance (data not shown). Thus, ICE-βox increases *L. pneumophila* fitness by protecting cells from β-lactam antibiotics, hydrogen peroxide, and bleach, three sources of oxidative stress.

External oxidative stress does not induces ICE-βox excision and transfer. For some elements, ICE excision and transfer are controlled by regulators encoded on the island that respond to the same stressful stimuli against which the ICE protects (14). Since ICE-βox conferred resistance to agents known to inflict oxidative stress (Fig. 2), we investigated whether these treatments induce ICE-βox mobility or transfer. *L. pneumophila* ICE-βox donor cells were exposed for 1 h to oxacillin, penicillin, or hydrogen peroxide and then assayed for excision and transfer. ICE-βox excision was not significantly induced after the 1-h exposure to these oxidants, as judged by qPCR assays on isolated DNA (see Fig. S3A in the supplemental material). Even across a range of chemical concentrations and mating periods, transfer rates of ICE-βox from treated cells did not differ significantly from control levels (see Fig. S3B). Accordingly, these oxidative stresses do not appear to stimulate ICE-βox mobility.

ICE-βox confers protection to *L. pneumophila* in stringent macrophages. In human lungs, macrophages are the opportunistic host for *L. pneumophila*. Since ICE-βox increased resistance to oxidative stress *in vitro*, we next tested whether ICE-βox enhances protection in macrophages, immune cells that generate reactive

oxygen species to kill microbes (15). A/J mice are permissive to *L. pneumophila* infection due to mutations in their Naip5 cytosolic surveillance protein (16). After 48 h in primary macrophages derived from A/J bone marrow cells, the yield of ICE-βox donor and transconjugant bacteria increased ~100-fold, whereas the recipient strain was significantly impaired (Fig. 3A). As a more stringent test of the impact of ICE-βox on *L. pneumophila* virulence, we next stimulated permissive A/J macrophages with gamma interferon (IFN-γ). Activated A/J macrophages killed the recipient strain that lacked ICE-βox, whereas the donor and transconjugant strains that harbored the ICE survived (Fig. 3B). We next tested whether ICE-βox increased *L. pneumophila* fitness in macrophages of Naip5$^{+/+}$ C57BL/6 mice, which are resistant to *L. pneumophila* infection (16, 17). During the first 24 h of infection of C57BL/6 bone marrow-derived macrophages, strains that carry ICE-βox replicated within macrophages ~10-fold more efficiently than did those that lacked the element ($P < 0.005$) (Fig. 3C). The fate of the intracellular bacteria was confirmed by examining the infected macrophages by fluorescence microscopy. At 24 h after infection, ICE-βox-lacking recipient bacteria were degraded, as evident from the particulate *L. pneumophila* antigen scattered throughout the cell (Fig. 3D and E). In contrast, multiple ICE-βox-containing donor and transconjugant bacteria resided within tight replication vacuoles (Fig. 3E), consistent with their increased yield (Fig. 3C). Therefore, ICE-βox enhances *L. pneumophila* infection and persistence within hostile mouse macrophages.

ICE-βox protection in macrophages is NADPH oxidase dependent. To test directly whether ICE-βox increases resistance to phagocyte oxidases, we compared *L. pneumophila* growth in macrophages that encode or that lack a component of the NADPH oxidase. For this purpose, we exploited a pair of BALB/c mouse derivative J774 wild-type (WT) and *phox* mutant macrophage cell lines (18). Infection of WT J774.16 cells by *L. pneumophila* strains that do or do not carry ICE-βox revealed a pattern similar to that observed for the infections of A/J and C57BL/6 primary macrophages. The yield of *L. pneumophila* strains that carry ICE-βox increased ~10-fold within the first 24 h, compared to a <2-fold increase in CFU for bacteria that lack the element (Fig. 3F). In contrast, after infection of J774.D9 *phox* mutant cells, all strains replicated >50-fold, regardless of their ICE-βox status (Fig. 3G). Therefore, ICE-βox increases *L. pneumophila* fitness within macrophages that encode NADPH oxidase.

DISCUSSION

ICEs contribute virulence and fitness traits to a wide array of pathogens, including *Vibrio cholerae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Here, we describe a transposable element named ICE-βox to emphasize its capacity to protect *L. pneumophila* from β-lactam antibiotics and oxidative stresses. Because aerosolization of environmental *L. pneumophila* is a prerequisite for infection (19), microbial factors that increase extracellular survival may also contribute to transmission to humans. Given the capacity of ICEs to transfer between nosocomial pathogens and to increase microbial fitness, the impact of water decontamination protocols on the incidence of human disease caused by *L. pneumophila* warrants analysis.

Although strain Philadelphia-1 is the only one of the eight *L. pneumophila* strains sequenced to date that carries ICE-βox, homology searches revealed that all eight strains contain the 43-bp *att* site for integration. Thus, it is likely that ICE-βox can spread among *L. pneumophila* strains. Loss of the element during derivation of the JR32 strain from the Philadelphia-1 clinical isolate (9) is consistent with the idea that ICE-βox is readily mobilized under certain environmental conditions. Exploratory experiments with *Escherichia coli*, *V. cholerae*, and *P. aeruginosa* indicate that *L. pneumophila* can transfer ICE-βox to other pathogens, but the element is not stably maintained, as judged by loss of its antibiotic resistance marker during subsequent passage of the respective transconjugant strains (data not shown). These genetic observations suggest that chromosomal maintenance of ICE-βox requires conserved *att* sites (Fig. 1A), a sequence not detected by *in silico* analysis of *E. coli*, *V. cholerae*, and *P. aeruginosa* or other non-*L. pneumophila* species.

To activate transcription of their genes, ICEs often integrate the regulatory circuitry carried on the element with core bacterial response systems, such as the SOS response (2). The ICE-βox region is rich with putative regulators, including homologs of the global regulators *csrA* and *lexA*, a small noncoding RNA located adjacent to the *att* sites, and putative diversity-generating retroelements (DGR) that alter expression of certain *L. pneumophila* traits (20). Indeed, the homologous region of ICE Trb-1 in *L. pneumophila* strain Corby has been shown by mutational analysis to regulate site-specific excision (3, 4). Insight into the regulation of ICE-βox cargo gene expression can be gained by molecular genetic analysis of this class of genes.

Among the 38 predicted cargo genes carried by ICE-βox are a number of stress response enzymes (8). Near its 3′ end is a putative oxidative stress resistance operon that includes two copies of the genes for detoxifying enzyme methionine sulfoxide reductase A and B (*msrAB*), as well as a cytochrome *c* oxidase (*lpg2100*) and an alkyl hydroperoxide reductase (*ahp*) homolog. The *msrAB* locus is of particular interest since in *Staphylococcus aureus msrA* transcription is upregulated in response to oxacillin (21). Additionally, *msrA* protects *E. coli* from hydrogen peroxide-induced killing (22) and increases tolerance of the intracellular *Mycobacterium smegmatis* to macrophage NADPH oxidase (23). Likewise, *ahp* homologs equip *Salmonella* species to scavenge free hydrogen peroxide and survive macrophage infection (24). Given their contribution to virulence in other pathogens, these enzymes are primary candidates to investigate the biochemical mechanism of protection by ICE-βox. Since the core chromosome of *L. pneumophila* also carries *msrA* and *ahpCD* genes, we speculate that a higher level of expression from the additional copies on ICE-βox increases *L. pneumophila* resistance to oxidative stress.

L. pneumophila persists in water supplies likely due to its ability to reside within both hardy biofilms and predatory amoebae (25). We did not detect any differences in the abilities of ICE-βox-containing and -lacking strains to replicate within *Acanthamoeba castellanii* (data not shown). Nevertheless, ICE-βox may contribute to increased survival in other aquatic phagocytes, as *L. pneumophila* is known to infect numerous amoeba species whose permissiveness varies (26, 27).

A remarkable finding is that samples from hospitals, schools, and spas contained a higher density of *Legionellae* than did their source water (28), suggesting that, relative to the natural environment, engineered water systems offer some advantage to this species. Typical hospital disinfectant regimens generate reactive chlorine species (RCS) oxidants from bleach, monochloramine, or chlorine dioxide (29). However, infection control treatments reduce but do not eliminate the pathogen (28), implying that a sub-

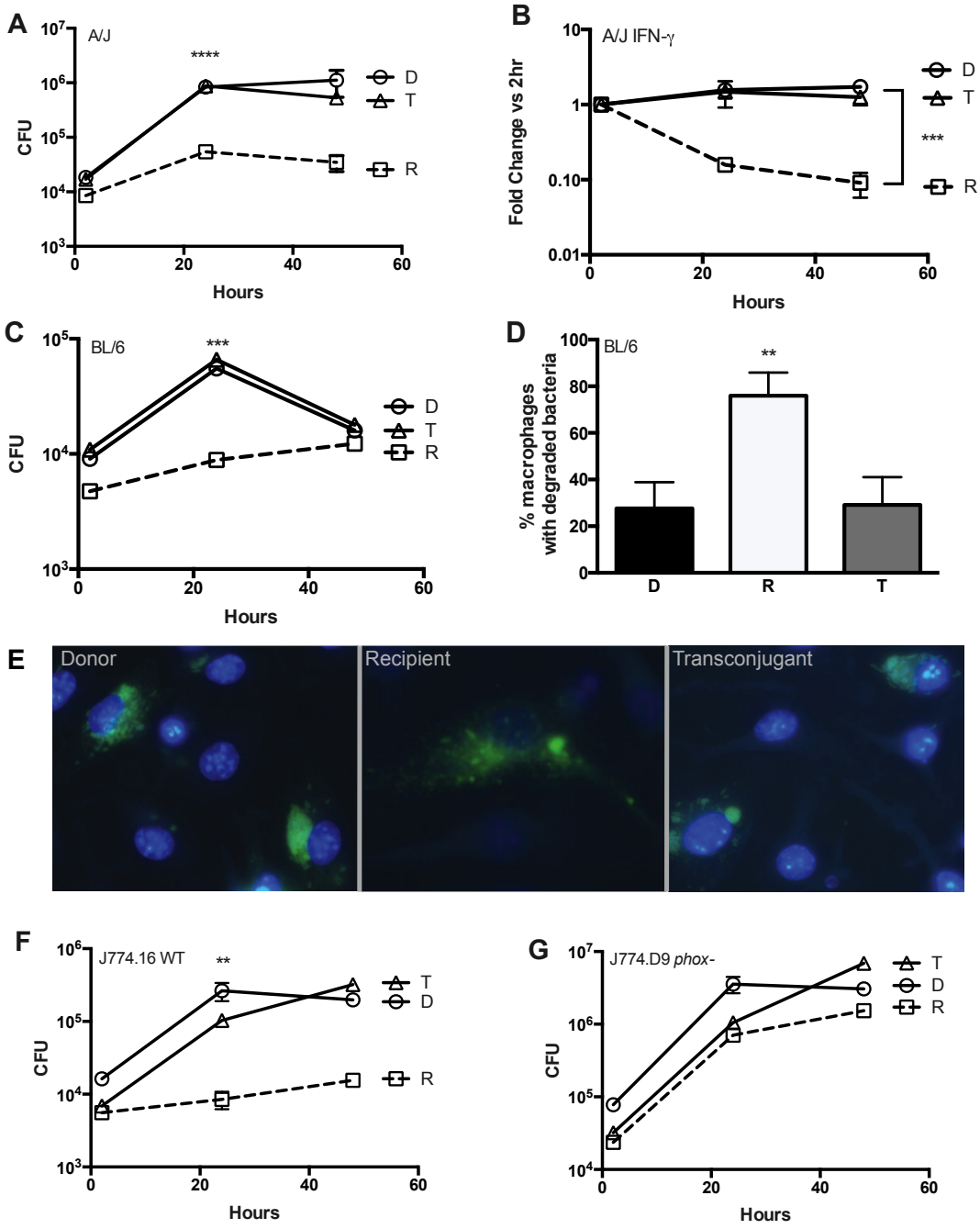

FIG 3 ICE-βox confers protection to *Legionella* in resistant macrophages. (A) ICE-βox increases fitness in permissive A/J macrophages. Macrophages were infected at an MOI of 1 with *L. pneumophila* strains that contain or lack ICE-βox. Shown are mean CFU ± SEM calculated at the times shown from triplicate samples in one experiment representative of three others. *t* tests indicate that growth differences between ICE-βox-containing and -lacking strains are significant (****, $P < 0.001$). (B) ICE-βox increases *L. pneumophila* persistence in activated A/J macrophages. Macrophages were activated with 100 U IFN-γ and were infected at an MOI of 1 with *L. pneumophila* strains that contain or lack ICE-βox. Shown is the mean ± SEM fold change in CFU at the times shown postinfection relative to the 2-h time point calculated from triplicate samples in one experiment representative of three others. *t* tests indicate that differences between ICE-βox-containing and -lacking strains are significant (***, $P < 0.005$). (C) ICE-βox increases *L. pneumophila* persistence in resistant C57BL/6 (BL/6) macrophages. After infection of bone marrow-derived macrophages as described in panel A, mean CFU ± SEM was calculated from three replicates in one experiment representative of three others. *t* tests indicate that differences between ICE-βox-containing and -lacking strains are significant (***, $P < 0.005$). (D and E) ICE-βox protects *L. pneumophila* from degradation in BL/6 macrophages. Twenty-four hours after infection, bacterial integrity was visualized by immunofluorescence microscopy using *L. pneumophila*-specific antibody (green) and the DNA stain DAPI (blue). Shown are the mean percentage ± SEM of macrophages that contained degraded bacteria calculated from three replicates in one experiment representative of three others. (E) Representative images of infected BL/6 macrophages are shown. *t* tests indicate that differences between ICE-βox-containing and -lacking strains are significant (**, $P < 0.01$). (F and G) ICE-βox's protective effects are dependent on NADPH oxidase. WT (F) or NADPH oxidase mutant (G) J774 cell lines were infected at an MOI of 1 with *L. pneumophila* strains containing or lacking ICE-βox. Shown are mean CFU ± SEM calculated from three replicates in one experiment representative of three others. *t* tests indicate that differences between ICE-βox-containing and -lacking strains are significant in WT macrophages (**, $P < 0.01$).

population of *L. pneumophila* is resistant to or protected from these chemicals. Indeed, in a survey of 209 samples from hospitals in Paris, France, use of continuous chlorination protocols correlated with an increase in the burden of pathogenic serogroup 1 *L. pneumophila* in their engineered water systems (30). Our findings show that ICE-βox promotes survival and replication in bleach concentrations comparable to that typical of drinking water (13). Given the widespread use of ROS and RCS in water decontamination procedures and the conservation of the *att* sites among *L. pneumophila* strains, the potential for spread of disinfectant resistance among populations of this opportunistic aquatic pathogen merits consideration.

Beyond increasing fitness in the environment, resistance to oxidative stress also enhances virulence. Production of ROS is a primary mechanism of the macrophage antibacterial response. Bacterial entry induces cellular NADPH oxidase to generate superoxide and hydrogen peroxide, which myeloperoxidase enzymes subsequently convert to the reactive chloride species HOCl (31, 32). Here, we have shown that *L. pneumophila* strains that acquire resistance to chlorine and oxidative stress also become more virulent in macrophages. Given this transposable element's ability to increase the fitness of planktonic bacteria under a variety of oxidizing conditions both *in vitro* and in macrophages, we postulate that ICE-βox increases the burden and virulence of pathogenic *L. pneumophila* in natural and engineered environments.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and reagents. *L. pneumophila* strain Philadelphia-1 derivatives JR32 and Lp02, a thymidine auxotroph, were cultured at 37°C in *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES; Sigma)-buffered yeast extract (AYE) broth supplemented with 100 μg/ml thymidine (Sigma). To quantify CFU, aliquots were plated on ACES-buffered charcoal-yeast extract agar (CYE) supplemented with 100 μg/ml thymidine (CYET) and incubated at 37°C for 4 days (33). Bacteria obtained from CYET were cultured overnight in AYET and then diluted and cultured overnight to obtain cells in lag (L; optical density at 600 nm [OD_{600}] of 0.5), exponential (E; OD_{600} of 1.2 to 1.8), late exponential (LE; OD_{600} of 2.5), or post-exponential (PE; OD_{600} of 3.2 to 3.5) phase (34). *E. coli* strains DH5α and DY330 were cultured under standard laboratory conditions at 37°C and 30°C, respectively. Penicillin G, oxacillin, gentamicin, chloramphenicol (Cam), and hydrogen peroxide were all purchased from Sigma. Bleach (Austin A-1; 6.15% NaOCl, 5.25% free chlorine) was purchased from Fisher Scientific. For bleach experiments, a new bottle was opened and diluted first in water and then in AYET for use in growth experiments. Chlorine content in AYET broth was quantified using chlorine test strips (Hach) and estimated to be at a final concentration of 0.5 ppm.

Mice. Six- to 8-week-old female A/J and C57BL/6 mice were purchased from Jackson Laboratories. Mice were housed in the University Laboratory Animal Medicine Facility at the University of Michigan Medical School under specific-pathogen-free conditions. The University Committee on Use and Care of Animals approved all experiments conducted in this study.

Generation of marked ICE-βox strains. The marked ICE-βox strain was generated by recombineering. The noncoding region between *lpg2110* and *lpg2111* was amplified by PCR using primers 5′ GATAGCAGCATG TTTACTAGTCGG 3′ and 5′ CGCATAACAAAGCGGCGC 3′ and cloned into pGEM-T Easy (Promega). After electroporation into *E. coli* strain DY330, λ-red recombinase replaced the corresponding ICE-βox noncoding region with a chloramphenicol (Cam) resistance cassette as described previously (35). The recombinant alleles were transferred to strain Lp02 via natural transformation to generate strain MB1353. Insertion of the Cam cassette was confirmed by selection on CYET-Cam and by PCR. Markerless deletions were created by electroporating pBSFlp into marked strains and selecting for the activity of the Flp recombinase to generate MB1357 and MB1358. Clones were screened for loss of the Cam cassette by PCR and for loss of pBSFlp by plating on selective medium.

Conjugation. To analyze ICE-βox transfer by conjugation, first the marked Lp02 donor strain MB1353 or the Lp02 control plasmid strain MB1326 was cultured to either E or PE phase, and the JR32 recipient strain MB1354 was cultured to PE phase. Next, 10^9 donor cells were mixed with 10^{10} recipient cells on 0.22-μm filters placed on prewarmed CYET agar plates with or without DNase I (1 μg/ml) and incubated for 2 h at 37°C as described previously (36). The mating mixture was harvested by suspending the filters in phosphate-buffered saline (PBS) and vortexing. Serial dilutions were plated on CYET-Cam (5 μg/ml) to select for donors or CYE-Cam to select for transconjugants. Conjugation efficiency was calculated as CFU transconjugants/CFU donor cells. To test the impact of particular stresses on conjugation efficiency, donor cells were pretreated in AYET for 1 to 2 h at 37°C with sublethal concentrations of penicillin G (5 to 10 μg/ml), oxacillin (50 to 100 μg/ml), or H_2O_2 (1 to 2 mM).

Excision. Episomal ICE-βox was detected by a PCR assay using template DNA isolated from *L. pneumophila* colonies and specific primers (see Table S1 in the supplemental material) to amplify either the chromosomal junction of ICE-βox or the junction of the episomal form as described previously (10). PCR products were column purified (QIAquick; Qiagen) and sequenced using an Applied Biosystems Model 3730 XL sequencer. Integrated and excised forms of ICE-βox were quantified by qPCR using specific primers (see Table S1) and 25 μg/ml template DNA prepared from strain Philadelphia-1 (Wizard SV genomic kit; Promega) either cultured to different growth stages or treated with penicillin, oxacillin, or H_2O_2 as described above. Reactions were carried out using a Bio-Rad iCycler and SYBR green (Bio-Rad) according to the manufacturer's protocol. As an internal reference, the *rpoS* gene was used to normalize input DNA. Log_2-transformed values of the mean ± standard error of the mean (SEM) fold change of episomal/chromosomal amplicons were calculated from three experiments performed in triplicate.

Phenotype microarrays and growth curves. A phenotype microarray screen was performed using PMA plates PM1 and PM12 from Biolog. Marked E-phase ICE-βox donor MB1353, transconjugant MB1352 or MB1355, and recipient JR32 MB1354 cultures were diluted in AYET to an OD_{600} of 0.2, and then 150 μl was transferred per well of each Biolog plate. Plates were incubated at 37°C with shaking, and every 3 h, the OD_{600} was determined using a plate spectrophotometer. Results of the Biolog screen were verified by growth curve assays. Strains were cultured in AYET to E phase and diluted, and cell density was normalized to an OD_{600} of 0.2 for triplicate samples. Bleach was diluted in water and added to the cell suspension to a final concentration of 55 ppm. Growth was quantified hourly as OD_{600} using a Bioscreen growth curve analyzer set at 37°C and shaking at medium amplitude. Stress resistance was assessed by quantifying CFU after treating triplicate E-phase samples subcultured in AYET to an OD_{600} of 0.8 for 6 h with penicillin, oxacillin, and H_2O_2 as described above. Mean ± SEM fold change was calculated as CFU at 6 h/CFU at 0 h from three independent experiments. Although the Cam cassette modestly increases oxacillin resistance, the resistance of two transconjugant strains cured of the Cam marker (MB1357 and MB1358) was verified to be significantly greater than that of the JR32 recipient (see Fig. S2B in the supplemental material). As an additional specificity control, recipient strains transformed with the plasmid-borne Cam resistance cassette (MB1351) were analyzed in each experiment that used marked strains.

Intracellular growth and immunofluorescence microscopy. Growth of *L. pneumophila* strains containing or lacking ICE-βox (donor MB1353, transconjugant MB1352, and recipient MB1351) was assessed in bone marrow-derived macrophages from C57BL/6 or A/J mice (Jackson Laboratories) as described previously (37). Means ± standard deviations (SD) were calculated from triplicate samples. Microscopy was performed by culturing 2.5×10^5 macrophages on 12-mm glass coverslips overnight prior to infection with PE-phase bacteria at a multiplicity of infection

(MOI) of 1. After 24 h, cultures were fixed and stained as described previously (38) using a 1:50 dilution of anti-*L. pneumophila* primary antibody obtained from mouse monoclonal hybridoma cell line CRL-1765 (ATCC) and a 1:1,000 dilution of anti-mouse IgG antibody conjugated to Oregon Green (Molecular Probes). The DNA stain DAPI (4′,6-diamidino-2-phenylindole) was purchased as ProLong Gold antifade reagent (Molecular Probes) and included in the mounting medium. NADPH oxidase experiments were performed using a pair of cell lines derived from the BALB/c mouse background. J774.16 (WT) and J774.D9 *phox* (deficient in gp91 subunit of NADPH oxidase) cells were infected as described above (18).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01091-14/-/DCSupplemental.

Figure S1, TIF file, 1.6 MB.
Figure S2, TIF file, 1.2 MB.
Figure S3, TIF file, 1.4 MB.
Table S1, DOCX file, 0.1 MB.

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