

The Bacterial *iprA* Gene Is Conserved across *Enterobacteriaceae*, Is Involved in Oxidative Stress Resistance, and Influences Gene Expression in *Salmonella enterica* Serovar Typhimurium

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ABSTRACT

The *iprA* gene (formerly known as *yaiV* or STM0374) is located in a two-gene operon in the *Salmonella enterica* serovar Typhimurium genome and is associated with altered expression during spaceflight and rotating-wall-vessel culture conditions that increase virulence. However, *iprA* is uncharacterized in the literature. In this report, we present the first targeted characterization of this gene, which revealed that *iprA* is highly conserved across *Enterobacteriaceae*. We found that *S. Typhimurium*, *Escherichia coli*, and *Enterobacter cloacae* $\Delta iprA$ mutant strains display a multi-log-fold increase in oxidative stress resistance that is complemented using a plasmid-borne wild-type (WT) copy of the *S. Typhimurium iprA* gene. This observation was also associated with increased catalase activity, increased *S. Typhimurium* survival in macrophages, and partial dependence on the *katE* gene and full dependence on the *rpoS* gene. Our results indicate that IprA protein activity is sensitive to deletion of the N- and C-terminal 10 amino acids, while a region that includes amino acids 56 to 80 is dispensable for activity. RNA sequencing (RNA-Seq) analysis revealed several genes altered in expression in the *S. Typhimurium* $\Delta iprA$ mutant strain compared to the WT, including those involved in fimbria formation, *spvABCD*-mediated virulence, ethanolamine utilization, the phosphotransferase system (PTS) transport, and flagellin phase switching from FlgB to FlgC (likely a stochastic event) and several genes of hypothetical or putative function.

IMPORTANCE

Overall, this work reveals that the conserved *iprA* gene measurably influences bacterial biology and highlights the pool of currently uncharacterized genes that are conserved across bacterial genomes. These genes represent potentially useful targets for bacterial engineering, vaccine design, and other possible applications.

Many large-scale genomic studies, virulence factor identification assays, and whole-genome expression analyses have revealed bacterial genes that are uncharacterized but are associated with gene expression changes in different environments that can affect virulence and stress survival (see references 1–8 and several others). These studies reveal a pool of genes that are not understood or characterized but whose importance is highlighted due to how they were identified and, in some cases, to high conservation across bacterial genomes. The *iprA* gene (previously known as *yaiV* or STM0374) is located in a two-gene operon in the *Salmonella enterica* serovar Typhimurium genome and has been observed to display altered expression during spaceflight and under rotating-wall-vessel (RWV) culture conditions, which increase virulence (6, 7). In these studies, microarray analysis revealed that *S. Typhimurium iprA* was upregulated in cultures inoculated and grown during spaceflight (compared to identically grown ground controls) and in cultures grown under low-fluid-shear RWV conditions (compared to RWV controls in which low-fluid-shear conditions were disrupted) (6, 7). Interestingly, both spaceflight and RWV growth conditions increase the virulence of *S. Typhimurium* assayed using murine infection models (6, 9, 10). However, to our knowledge, the *iprA* gene is uncharacterized in the literature beyond these observations. Overall, little is known about the seven genes in the 8.9-kb locus between *hemB* and *ddlA* in the *S. Typhimurium* genome, which include *yaiU*, *iprA*, *ampH*, *sbmA*, *yaiW*, *yaiY*, and *yaiZ* (11). The *ampH* gene has been shown to be decreased in expression in the presence of butyrate (12). The *sbmA* and *yaiW* genes are cotranscribed and encode membrane proteins that affect sensitivity to antimicrobial peptides (13). The func-

tional roles of the *yaiU*, *iprA*, *yaiY*, and *yaiZ* genes were hitherto unknown. In this report, we present the first targeted systematic characterization of the *iprA* gene, which reveals that *iprA* is highly conserved and found across *Enterobacteriaceae* genomes. Our results indicate a role for *iprA* in bacterial oxidative stress resistance across genera, where the deletion of *iprA* is associated with increased resistance to hydrogen peroxide, increased catalase activity, and increased *S. Typhimurium* survival in macrophage cells. We found the increased resistance to hydrogen peroxide to be partially dependent on the *katE* gene and fully dependent on the *rpoS* gene. We also found that deletion of *S. Typhimurium iprA* results in alteration of *S. Typhimurium* gene expression, including that of fimbrial genes, the ethanolamine utilization operon, plasmid-borne *spvABCD* genes, phosphotransferase system (PTS) transport genes, phage genes, and many putative or hypothetical

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genes. Thus, *iprA* is a previously unrecognized target gene for possible use in bacterial engineering, vaccine design, and other approaches where *iprA*-regulated phenotypes or associated gene pathways have a need to be manipulated for different applications. Based on this work, we introduce the name *iprA* (inhibitor of hydrogen peroxide resistance) for this gene. In addition, the results highlight undiscovered gene functions that reside in the pool of currently uncharacterized genes that are conserved across genera in bacterial genomes. We also discuss the role that *iprA* might play as a gene whose deletion results in increased resistance to oxidative stress.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used in this study are listed in Table 1. The construction of all deletion mutant strains utilized standard recombineering (14, 15), with PCR products obtained using the DNA primers listed in Table S1 in the supplemental material. The template plasmid pKD3, pKD4, or pJW102 was used as indicated in Table S1 to amplify the chloramphenicol resistance (Cm^r), kanamycin resistance (Km^r), or spectinomycin resistance (Sp^r) marker for insertion, respectively, and the marker was subsequently removed using flippase (FLP) recombinase expressed from plasmid pCP20 (14, 15). Deletion endpoints were internal to the indicated gene open reading frames (ORFs) using the homology in the primers displayed in Table S1. Molecular verification of all mutations was performed via PCR, with primers amplifying specific products indicative of each deletion. Deletion mutations were transferred between *S. Typhimurium* strains using standard P22 HT/*int* phage transduction, as described previously (16). The transfer of deletion mutations between *Escherichia coli* strains was performed using purified donor chromosomal DNA transformed into recipient strains containing plasmid pKD46-RecA, as described previously (17). Construction of the *S. Typhimurium* $\Delta N10$ and $\Delta C10$ mutant strains was performed via genome recombineering that resulted in the in-frame removal of the very N-terminal and C-terminal 10 amino acids of the IprA protein, respectively. These mutations were verified via sequencing. The *S. Typhimurium iprA* wild-type (WT) allele and the $\Delta 56-80$ allele (which encodes a deletion of amino acids 56 to 80) were obtained via gene synthesis (Genewiz, Piscataway, NJ) and subcloned into the plasmid vector pBAD18 (18) at the *Xma*I site, using standard cloning procedures. These gene clones were verified via sequencing. Strains were grown using LB (Lennox) medium supplemented with the following antibiotics when necessary: ampicillin, 200 μ g/ml; chloramphenicol, 10 μ g/ml; kanamycin, 50 μ g/ml; and spectinomycin, 125 μ g/ml.

Sequence analysis. The IprA homologs from the indicated Gram-negative species were identified using BLAST search analysis with *S. Typhimurium* IprA (GenBank accession no. AAL19328) as the query (19). Homologs were aligned using CLUSTAL W analysis, as previously described (20, 21). Synteny analysis was performed using standard gene visualization tools via the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>).

Oxidative stress, catalase, macrophage survival, and *Caenorhabditis elegans* assays. Oxidative stress assays were performed by adding hydrogen peroxide (Sigma, St. Louis, MO) to aliquots of cultures that had been grown for 4 h after inoculation from an overnight culture into fresh broth, as described previously (22–25). Cultures for the assays were used at an optical density at 600 nm (OD_{600}) of 0.5 to 0.6, and graphs of the CFU amounts used in a typical experiment serve to show that samples were at equivalent CFU at the time of the stress assay (see Fig. S1 in the supplemental material). Survival was routinely assayed over 2 h using a hydrogen peroxide concentration of 70 mM; however, lower levels were tested for the $\Delta katE$, $\Delta katG$, $\Delta katN$, $\Delta rpoS$, and Δdps background mutant strains due to hypersensitivity. Those levels were as follows: $\Delta katE$ mutant, 27 mM; $\Delta katG$ mutant, 35 mM; $\Delta katN$ mutant, 35 mM; $\Delta katE \Delta katG \Delta katN$ mutant, 17.5 mM; $\Delta rpoS$ mutant, 9 mM; and Δdps mutant, 17.5 mM. The

TABLE 1 Strains and plasmids used in this study

Plasmid or strain	Species	Reference or source
Strains		
$\chi 3339$	<i>S. Typhimurium</i>	37
$\chi 3339 \Delta U-Z$	<i>S. Typhimurium</i>	This study
$\chi 3339 \Delta H-Z$	<i>S. Typhimurium</i>	This study
$\chi 3339 \Delta U-V$	<i>S. Typhimurium</i>	This study
$\chi 3339 \Delta U$	<i>S. Typhimurium</i>	This study
$\chi 3339 \Delta iprA$	<i>S. Typhimurium</i>	This study
$\chi 3339 \Delta katN$	<i>S. Typhimurium</i>	This study
$\chi 3339 \Delta katG$	<i>S. Typhimurium</i>	This study
$\chi 3339 \Delta katE$	<i>S. Typhimurium</i>	This study
$\chi 3339 \Delta oxyR$	<i>S. Typhimurium</i>	This study
$\chi 3339 \Delta rpoS$	<i>S. Typhimurium</i>	49
$\chi 3339 \Delta dps$	<i>S. Typhimurium</i>	This study
$\chi 3339 \Delta ahpC$	<i>S. Typhimurium</i>	This study
$\chi 3339 \Delta tsaa$	<i>S. Typhimurium</i>	This study
$\chi 3339 \Delta katN katG katE$	<i>S. Typhimurium</i>	This study
$\chi 3339 \Delta iprA \Delta katN$	<i>S. Typhimurium</i>	This study
$\chi 3339 \Delta iprA \Delta katG$	<i>S. Typhimurium</i>	This study
$\chi 3339 \Delta iprA \Delta katE$	<i>S. Typhimurium</i>	This study
$\chi 3339 \Delta iprA \Delta katN katG katE$	<i>S. Typhimurium</i>	This study
$\chi 3339 \Delta iprA \Delta rpoS$	<i>S. Typhimurium</i>	This study
$\chi 3339 \Delta iprA \Delta dps$	<i>S. Typhimurium</i>	This study
$\chi 3339 \Delta iprA \Delta ahpC$	<i>S. Typhimurium</i>	This study
$\chi 3339 \Delta iprA \Delta tsaa$	<i>S. Typhimurium</i>	This study
$\chi 3339 \Delta iprA \Delta oxyR$	<i>S. Typhimurium</i>	This study
$\chi 3339 \Delta N10$	<i>S. Typhimurium</i>	This study
$\chi 3339 \Delta C10$	<i>S. Typhimurium</i>	This study
ATCC 29629	<i>S. Typhimurium</i>	American Type Culture Collection
ATCC 29629 $\Delta iprA$	<i>S. Typhimurium</i>	This study
NTCT74	<i>S. Typhimurium</i>	50
NTCT74 $\Delta iprA$	<i>S. Typhimurium</i>	This study
MG1655	<i>E. coli</i>	51
MG1655 $\Delta iprA$	<i>E. coli</i>	This study
JA221	<i>E. coli</i>	52
JA221 $\Delta iprA$	<i>E. coli</i>	This study
TOP10	<i>E. coli</i>	Invitrogen
TOP10 $\Delta iprA$	<i>E. coli</i>	This study
ATCC 23355	<i>Enterobacter cloacae</i>	American Type Culture Collection
ATCC 23355 $\Delta iprA$	<i>E. cloacae</i>	This study
Plasmids		
pBAD18		18
pBAD18 + <i>iprA</i>		This study
pBAD18 + <i>iprA</i> $\Delta 56-80$		This study
pKD3		14
pKD4		14
pCP20		14
pJW102		15
pKD46-RecA		Nature Technology Corp., Lincoln, NE

oxidative stress data presented represent the mean and standard deviation of the results from at least four independent experiments, each plated in triplicate. Growth curve assays in LB broth culture were performed as described previously in the presence of 5 mM H_2O_2 to test inhibitory effects in this context (26). Bovine liver catalase protein (Sigma) was supplemented to solid medium at 140 μ g/ml in the experiment shown in Fig. 7 (27). Catalase activity assays were performed as described previously,

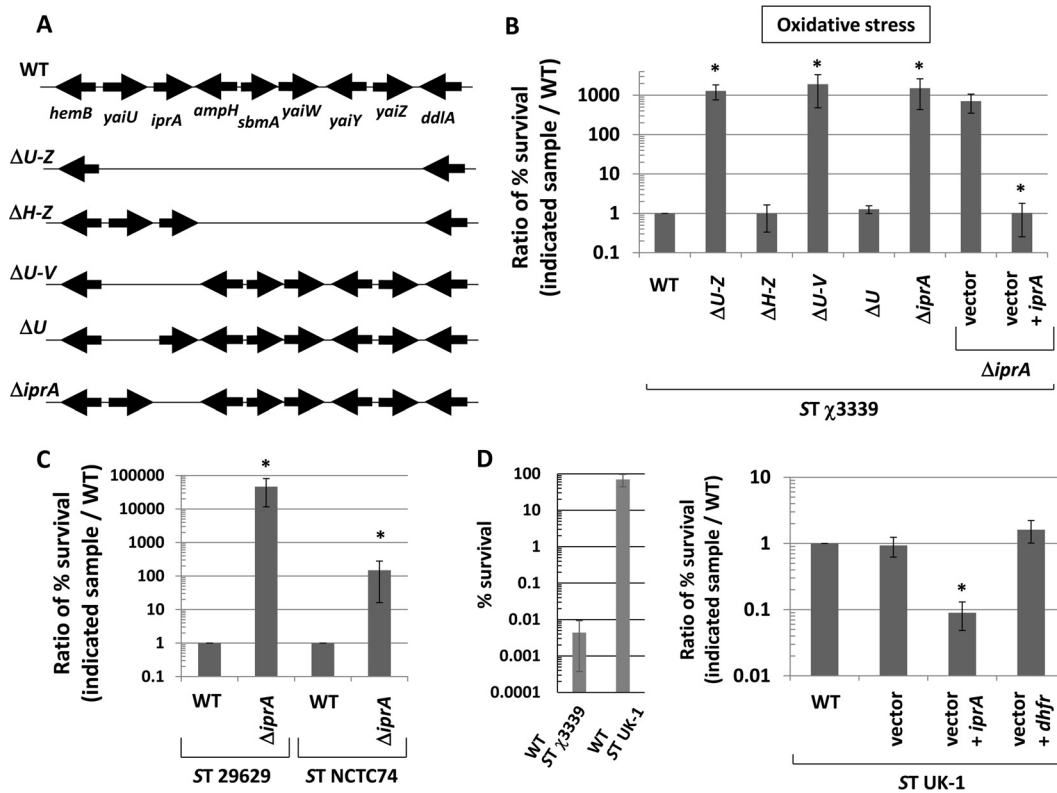


FIG 2 Role of the *iprA* gene in oxidative stress resistance in *S. Typhimurium* (ST). (A) Gene map between *hemB* and *ddlA* in the *S. Typhimurium* genome showing different deletion mutations constructed in this region. (B) Oxidative stress resistance assays performed on *S. Typhimurium* strain χ 3339 derivatives containing different mutations shown in panel A. The percent survival of bacteria after the addition of stress was obtained based on the initial CFU per milliliter before stress, and a ratio of the percent survival for each strain compared to the WT strain survival was calculated and plotted. (C) *S. Typhimurium* strains 29629 and NCTC74 containing $\Delta iprA$ mutations were compared to the corresponding WT strains in oxidative stress assays. (D) The *S. Typhimurium* strain UK-1 displays inherently high oxidative stress resistance in this study (as shown in the graph of oxidative stress assay results on the left). UK-1 strains containing plasmid vector, vector plus *iprA*, or vector plus the dihydrofolate reductase gene (*dhfr*) were tested for oxidative stress resistance. Data in each panel are shown as the mean plus standard deviation, and observed differences from the WT were found to be significant (as indicated by an asterisk) at a *P* value of <0.05 using a *t* test to compare the WT and the indicated mutant sample. For panels B and D, a *t* test comparison between the vector and the vector plus *iprA* was used.

the supplemental material). These results indicate that the *iprA* gene is conserved in *Enterobacteriaceae* across multiple genera and suggest evolutionary selection for the function of this gene in prokaryotic biology.

Our sequence analysis revealed a predicted catabolite activator protein (CAP) effector binding domain in IprA that spans amino acid residues 23 to 116 and aligns with the consensus CAP effector binding domain sequence at an E value of 3.52×10^{-8} (see Fig. S3 in the supplemental material). The effector domain acts to bind a molecule that triggers conformational change to influence DNA binding of the protein (such as the cyclic AMP [cAMP] molecule does to the CAP/cAMP receptor protein [CRP]). Our analysis also found that the IprA protein contains a predicted winged helix-turn-helix (wHTH) DNA-binding domain that spans amino acids 138 to 205, which aligns with the consensus sequence at an E value of 1.9×10^{-29} (see Fig. S4 in the supplemental material). The identification of these predicted domains suggests that IprA could be a DNA-binding protein whose binding activity is influenced or regulated by the binding of an effector molecule via the predicted CAP effector domain.

Phenotypic analysis of *S. Typhimurium* $\Delta iprA$ mutant strains. To determine the role of *iprA* and other nearby genes in bacterial biology, we constructed a series of deletions in the *S.*

Typhimurium genome that removed different combinations of the genes between *hemB* and *ddlA*, which included *yaiU*, *iprA*, *yaiW*, *yaiY*, and *yaiZ* (Fig. 2A). We compared the survival of these mutant strains to that of the isogenic wild-type strain under various environmental stresses (including acid, oxidative, thermal, and osmotic) in the virulent *S. Typhimurium* background χ 3339 (37). We found that all mutants containing a deletion of the *iprA* gene displayed enhanced survival of oxidative stress in this background at a level approximately 1,000-fold greater than that of the wild type (Fig. 2B). In addition, we set up growth curve assays with WT and $\Delta iprA$ mutant bacteria in the presence of inhibitory oxidative stress levels and found that the deletion of *iprA* resulted in enhanced survival under these conditions as well (see Fig. S5 in the supplemental material). The results of the acid, thermal, and osmotic stress assays were either inconclusive or displayed equivalent resistance levels in the mutant and wild-type strains (data not shown). The enhanced oxidative stress resistance phenotype of the *S. Typhimurium* $\Delta iprA$ mutant was fully complemented to the wild-type phenotype using a plasmid-borne copy of the *S. Typhimurium iprA* gene (Fig. 2B).

To determine if we could observe the $\Delta iprA$ mutant phenotype in other *S. Typhimurium* strains, we transferred the $\Delta iprA$ mutation to *S. Typhimurium* strains 29629 and NCTC74 and com-

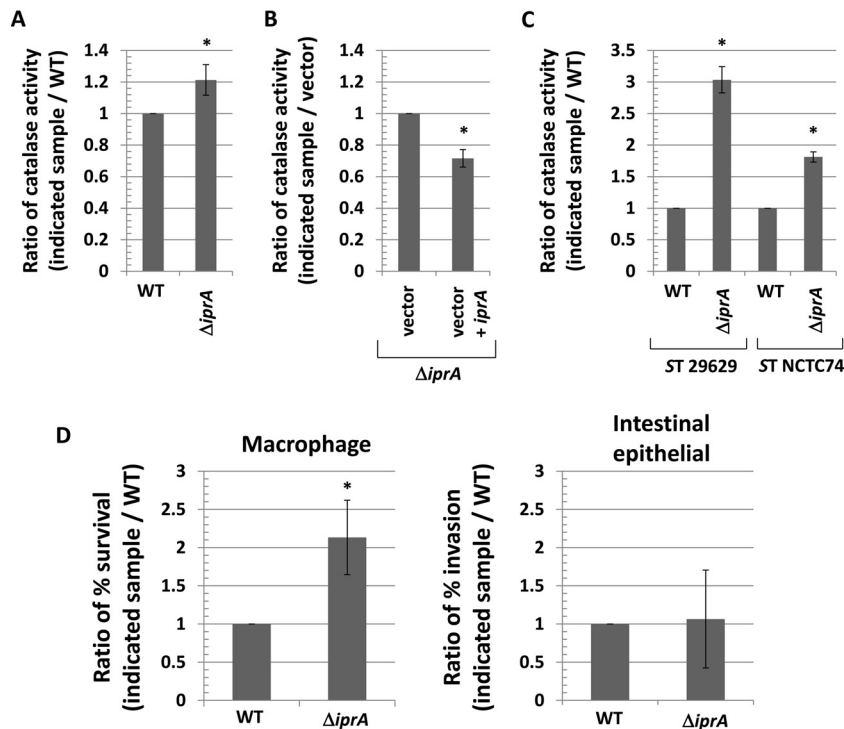


FIG 3 Increased catalase activity and survival in macrophages associated with $\Delta iprA$ mutation in *S. Typhimurium*. (A) *S. Typhimurium* $\chi 3339$ WT and $\chi 3339$ $\Delta iprA$ mutant strains were measured for catalase activity, and a ratio of the activity in each sample to the WT value was calculated and plotted. (B) *S. Typhimurium* $\chi 3339$ $\Delta iprA$ mutant strains containing plasmid vector or vector plus $iprA$ were measured for catalase activity, and a ratio of the activity in each sample compared to the vector strain activity was calculated and plotted. (C) *S. Typhimurium* strains 29629 and NTCT74 containing $\Delta iprA$ mutations were compared to each corresponding WT for catalase activity. (D) *S. Typhimurium* strain $\chi 3339$ WT and $\Delta iprA$ mutant strains were used to infect J774 macrophage cells and Int407 intestinal epithelial cells, and the percent survival (in macrophages) and percent invasion (for intestinal epithelial cells) were measured for each strain at 2 h postinfection. A ratio of these values for each sample to the values for the WT was calculated and plotted. Data in each panel are shown as the mean plus standard deviation, and observed differences from the WT were found to be significant (as indicated by an asterisk) at a *P* value of <0.05 using a *t* test to compare the WT and the indicated mutant sample or, for panel B, the vector and the vector plus $iprA$.

pared the corresponding mutant and WT strains in the oxidative stress assay (Fig. 2C). The 29629 and NTCT74 $\Delta iprA$ mutant strains displayed approximately 40,000- and 100-fold enhanced oxidative stress survival, respectively, compared to the cognate WT strain, indicating that this phenomenon is not strain specific. The *S. Typhimurium* universal killer strain UK-1 displays an inherently high resistance to oxidative stress in our hands compared to $\chi 3339$ (Fig. 2D), and we reasoned that the overexpression of $iprA$ in this strain would be predicted to decrease this resistance. When we expressed plasmid-borne $iprA$ in *S. Typhimurium* UK-1, the oxidative stress resistance decreased as predicted, and this was not observed in control strains containing plasmid vector alone or expressing a heterologous protein (dihydrofolate reductase [DHFR]) from the same vector (Fig. 2D).

To determine if the increased resistance to hydrogen peroxide-induced oxidative stress in the *S. Typhimurium* $\chi 3339$ $\Delta iprA$ mutant strain corresponded with an increase in catalase activity, we measured the catalase activity in the WT and $\Delta iprA$ $\chi 3339$ strains (Fig. 3A). We found that $\chi 3339$ $\Delta iprA$ displayed approximately 20% more catalase activity than the WT strain, and this activity was correspondingly reversed by a WT copy of the $iprA$ gene provided on a plasmid (Fig. 3B). We also measured the catalase activity in the *S. Typhimurium* 29629 and NTCT74 WT and $\Delta iprA$ mutant strains and found approximately 300% and 75% increases in catalase activity in the 29629 $\Delta iprA$ and NTCT74 $\Delta iprA$ mutant

strains compared to the cognate WT, respectively (Fig. 3C). Thus, the *S. Typhimurium* $\Delta iprA$ mutation results in both an increase in oxidative stress resistance and an increase in catalase activity.

To test if the *S. Typhimurium* $\Delta iprA$ mutation alters survival in macrophages (where reactive oxygen species aid in killing phagocytosed bacteria), we infected macrophage cells with *S. Typhimurium* $\chi 3339$ WT and $\Delta iprA$ mutant strains and assayed survival at 2 h postinfection (Fig. 3D). We found that the $\Delta iprA$ mutant strain displayed approximately 2-fold greater survival than the WT in macrophages (Fig. 3D). This is consistent with the increased survival observed for *S. Typhimurium* $\Delta iprA$ mutant strains in the oxidative stress assays described above. We also compared the $\chi 3339$ WT and $\Delta iprA$ mutant strains for the invasion of intestinal epithelial cells (another key virulence attribute of *S. Typhimurium*) but found equivalent levels of invasion of these strains into these cells (Fig. 3D). We measured the survival of *C. elegans* worms infected with either the *S. Typhimurium* $\chi 3339$ WT or $\Delta iprA$ mutant strain (see Fig. S6 in the supplemental material), and we found no difference in the survival of the worms infected with these strains.

***Escherichia coli* and *Enterobacter cloacae* $\Delta iprA$ mutant strains.** Given the strong conservation of the $iprA$ gene across *Enterobacteriaceae*, we constructed a deletion mutation of the $iprA$ gene in *Escherichia coli*, and we then compared the *E. coli* $\Delta iprA$ mutant strain to the corresponding WT strain for resistance to

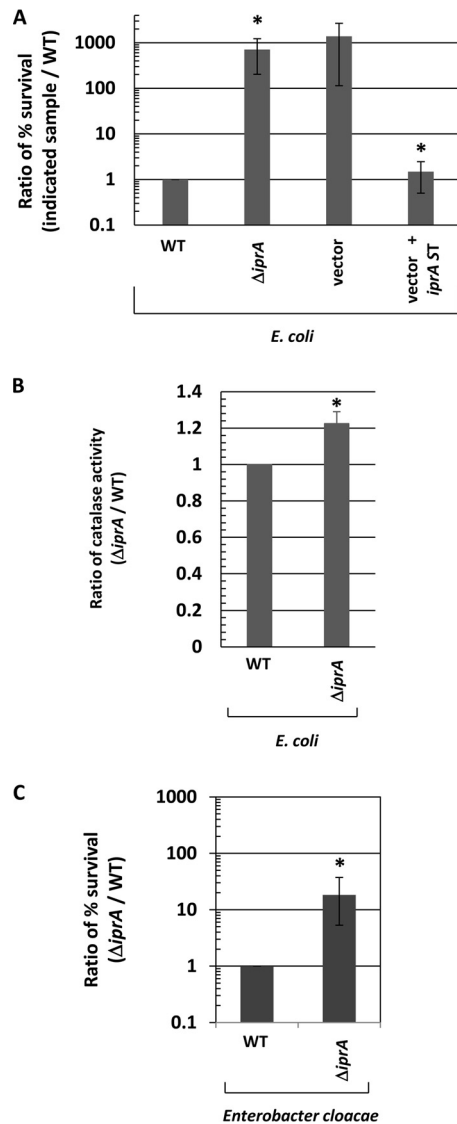


FIG 4 Characterization of *E. coli* and *E. cloacae* $\Delta iprA$ mutant strains. (A) A $\Delta iprA$ mutation was constructed in *E. coli* and transferred to *E. coli* strain TOP10. The percent survival of the $\Delta iprA$ mutant upon exposure to oxidative stress was measured and calculated for a ratio to the survival of the corresponding WT strain. TOP10 $\Delta iprA$ mutant strains containing plasmid vector and vector plus *S. Typhimurium* (ST) *iprA* were assayed for oxidative stress survival and compared to the corresponding WT strain. The *S. Typhimurium iprA* gene is the WT *iprA* gene cloned from *S. Typhimurium*. (B) Catalase activity was measured in the WT and *E. coli* TOP10 $\Delta iprA$ mutant strains and plotted as the ratio of the activity of each sample to that of the corresponding WT strain. (C) An $\Delta iprA$ mutation was constructed in *E. cloacae*, and the mutant was assayed for oxidative stress survival, as performed for the *E. coli* strains. Data in each panel are shown as the mean plus standard deviation, and the observed differences from the WT were found to be significant (as indicated by an asterisk) at a *P* value of <0.05 for all panels using a *t* test to compare the WT and the indicated mutant sample. For panel A, a *t* test comparison was also performed between vector and vector plus *iprA*.

oxidative stress (Fig. 4A). We found that the $\Delta iprA$ mutation resulted in an approximately 700-fold increase in oxidative stress resistance compared to the WT in the *E. coli* TOP10 background (Fig. 4A). We then transferred the $\Delta iprA$ mutation to two other *E. coli* strain backgrounds, MG1655 and JA221. We found that the

$\Delta iprA$ mutation resulted in approximately 80,000-fold and 20-fold increases in oxidative stress resistance compared to the WT in the MG1655 and JA221 backgrounds, respectively (see Fig. S7A in the supplemental material). We also measured the catalase activity of the above-mentioned *E. coli* $\Delta iprA$ mutant and WT strains and observed an increase in catalase activity for the $\Delta iprA$ mutant strains compared to the WT for each *E. coli* background (Fig. 4B; see also Fig. S7B). Since the homology between the *S. Typhimurium* and *E. coli* IprA proteins is high (71% identity, 86% similarity, *E* value = 10^{-108}), we reasoned that the *S. Typhimurium iprA* gene could be used for complementation analysis in the *E. coli* $\Delta iprA$ mutant strain. We found that the *S. Typhimurium iprA* gene provided on a plasmid complemented the *E. coli* $\Delta iprA$ mutation and restored oxidative stress resistance to WT levels in these strains (Fig. 4A; see Fig. S7C). In addition, we also constructed a $\Delta iprA$ mutation in *Enterobacter cloacae*, another Gram-negative species that contains the *iprA* gene (as shown in Fig. 1). The *E. cloacae* $\Delta iprA$ mutant strain displayed approximately 20-fold increased resistance to oxidative stress compared to the corresponding WT strain (Fig. 4C). These results clearly demonstrate the conserved nature of the *iprA* gene and its function across Gram-negative genera.

Deletion of IprA protein regions. To determine which regions of the IprA protein are essential for its activity, we constructed in-frame deletion mutations in the *iprA* gene that removed peptide sequences from the corresponding IprA protein. We constructed two mutations, $\Delta N10$ and $\Delta C10$, which removed 10 amino acids from the very N and C termini of the *S. Typhimurium* IprA protein, respectively (Fig. 5A). Both mutations abolished *iprA* activity but could be complemented to the WT phenotype using a WT copy of the *iprA* gene provided on a plasmid (Fig. 5B). In addition, we constructed a mutation that removed amino acids 56 to 80 from the *S. Typhimurium* IprA protein and served to delete a significant portion from the middle of the predicted CAP effector binding domain that includes the predicted ligand binding site (Fig. 5A). This mutation did not alter *iprA* gene activity (Fig. 5B). These analyses demonstrate that the N and C termini of the IprA protein are critical to its activity and sensitive to removal and that the portion of the predicted CAP effector binding domain comprising amino acids 56 to 80 is dispensable.

Role of catalase genes, *rpoS*, and other genes in the $\Delta iprA$ mutant phenotype. To learn what mechanistic pathways are involved in the increased oxidative stress resistance observed in the $\Delta iprA$ mutant strains, we constructed deletions in genes encoding catalase enzymes in *S. Typhimurium*: *katN*, *katG*, and *katE* (27). We constructed $\chi 3339$ double mutant strains that combined the $\Delta katN$, $\Delta katG$, or $\Delta katE$ mutation with the $\Delta iprA$ mutation and compared these strains to the corresponding single-catalase-mutation strain. We specifically wanted to determine if any of the catalase mutations altered the increase in oxidative stress resistance observed in the presence of the $\Delta iprA$ mutation. The presence of the $\Delta iprA$ mutation increased oxidative stress resistance of the $\Delta katN$ and $\Delta katG$ mutant strains approximately 1,000-fold (Fig. 6). However, in the $\Delta katE$ mutant background, the $\Delta iprA$ mutation increased oxidative stress resistance approximately 10-fold, indicating a role for the *katE* gene in this phenotype (Fig. 6). To see if the combined activity of the *katN* and *katG* genes could account for the remaining 10-fold resistance observed in the $\Delta katE$ $\Delta iprA$ mutant strain, we constructed a strain containing mutations in all three catalase genes and then transferred the $\Delta iprA$ mutation to

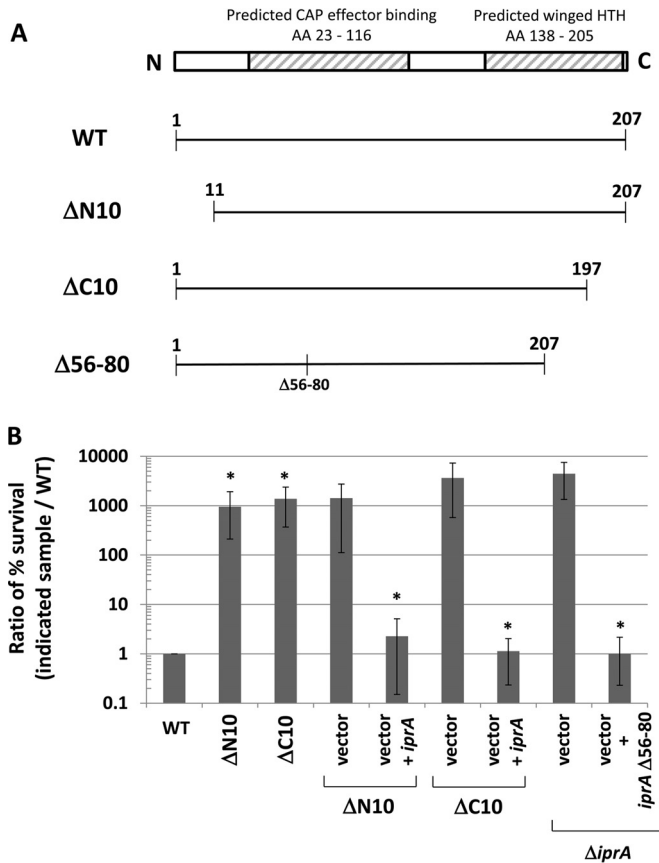


FIG 5 Deletion of IprA protein regions. (A) Diagram of WT IprA protein and the Δ N10, Δ C10, and Δ 56-80 mutant derivatives constructed in *S. Typhimurium* for this study. The Δ N10 and Δ C10 alleles remove the first 10 amino acids (AA) from the N terminus and last 10 amino acids from the C terminus from *S. Typhimurium* IprA, respectively. The Δ 56-80 allele contains a deletion of amino acids 56 to 80 located in the predicted CAP effector binding domain of the *S. Typhimurium* IprA protein. The Δ N10 and Δ C10 alleles were constructed in the chromosome, and the Δ 56-80 allele was constructed in a plasmid. (B) *S. Typhimurium* strain χ 3339 containing either the Δ N10 or Δ C10 mutation was tested for oxidative stress survival and compared to the isogenic WT strain, as described for previous experiments. The same mutant strains containing plasmid vector and vector plus *iprA* were also tested for oxidative stress survival and compared to the WT. The χ 3339 Δ iprA mutant strain containing plasmid vector or vector plus the *iprA* Δ 56-80 mutant was tested for oxidative stress survival in the same manner as the other strains. Data are shown as the mean plus standard deviation, and observed differences were found to be significant (as indicated by an asterisk) at a *P* value of <0.05 using a *t* test to compare either the WT and the indicated mutant or the vector and the vector plus *iprA*, as indicated.

this background. The results show that the Δ iprA mutation increased resistance 10-fold in this background, indicating that the Δ iprA mutant phenotype works through the *katE* catalase for part of this phenotype and is likely independent of the *katN* and *katG* genes (Fig. 6). We then transferred the Δ iprA mutation into an isogenic *S. Typhimurium* Δ rpoS background mutant strain to test for a role of the *rpoS* gene in the Δ iprA mutant phenotype. The *rpoS* gene encodes a sigma factor involved in the expression of several stress resistance genes, including the *katE* gene (38, 39). Interestingly, we found that the Δ iprA mutation did not increase oxidative stress resistance in the Δ rpoS mutant background, indicating that the *rpoS* gene is essential for the full Δ iprA mutant

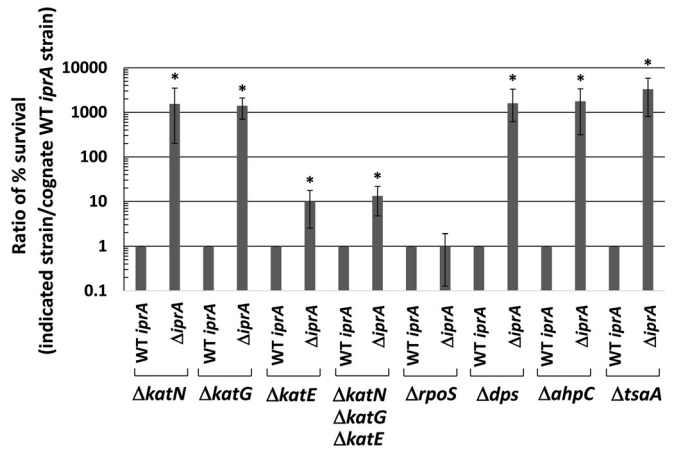


FIG 6 Role of catalase genes, *rpoS*, and other genes in the Δ iprA mutant phenotype. *S. Typhimurium* strain χ 3339 containing the indicated mutations was tested for oxidative stress survival, and each strain was compared to the corresponding strain containing the WT *iprA* allele. Data are shown as the mean plus standard deviation, and observed differences from cognate strains containing WT *iprA* were found to be significant (as indicated by an asterisk) at a *P* value of <0.05 using a *t* test.

phenotype (Fig. 6). This suggests that an additional RpoS-controlled factor plays a role in the Δ iprA oxidative stress resistance mutant phenotype. Therefore, we tested for a role of the *dps* gene in the Δ iprA mutant phenotype. The *dps* gene is regulated by RpoS and encodes a nonspecific DNA-binding protein that acts to protect bacterial cells against oxidative stress (40, 41). We constructed an *S. Typhimurium* Δ dps mutant strain and transferred the Δ iprA mutation into this strain. However, we found that the Δ iprA mutation increased oxidative stress resistance 1,000-fold in the Δ dps mutant strain, indicating that the *dps* gene is not involved in the Δ iprA mutant phenotype (Fig. 6).

Two *S. Typhimurium* genes encoding separate alkyl hydroperoxide reductases, which can scavenge and degrade hydrogen peroxide, are *ahpC* and *tsaA* (27). To test the involvement of these genes in the Δ iprA mutant phenotype, we constructed Δ ahpC and Δ tsaA mutations and tested them in combination with the Δ iprA mutation in oxidative stress assays (Fig. 6). We found that these mutations did not affect the Δ iprA mutant phenotype.

An *S. Typhimurium* Δ iprA Δ oxyR mutant displays a growth defect on solid medium. During our efforts to combine mutations in stress-related genes with the Δ iprA mutation as described above, we constructed a deletion mutation in the *S. Typhimurium* *oxyR* gene, which regulates the expression of oxidative stress genes, including *katG* (42, 43). However, when we combined the Δ oxyR and Δ iprA mutations, we observed that this strain was severely growth defective on solid medium (Fig. 7A). This growth defect was reversed by providing a WT copy of the *iprA* gene in the Δ oxyR Δ iprA mutant background (Fig. 7B). Interestingly, the growth defect for the Δ oxyR Δ iprA mutant strain was not observed in liquid medium, as its growth curve in broth was equivalent to that of isogenic control strains (see Fig. S8 in the supplemental material). Thus, it appears that the loss of both the *oxyR* and *iprA* genes can result in a cellular effect that causes a defect for normal colony formation on solid medium. We found that the presence of catalase protein supplemented into the agar medium reversed this phenotype, indicating that enhanced oxidative stress as present in solid medium plays a role in this observation (Fig. 7C).

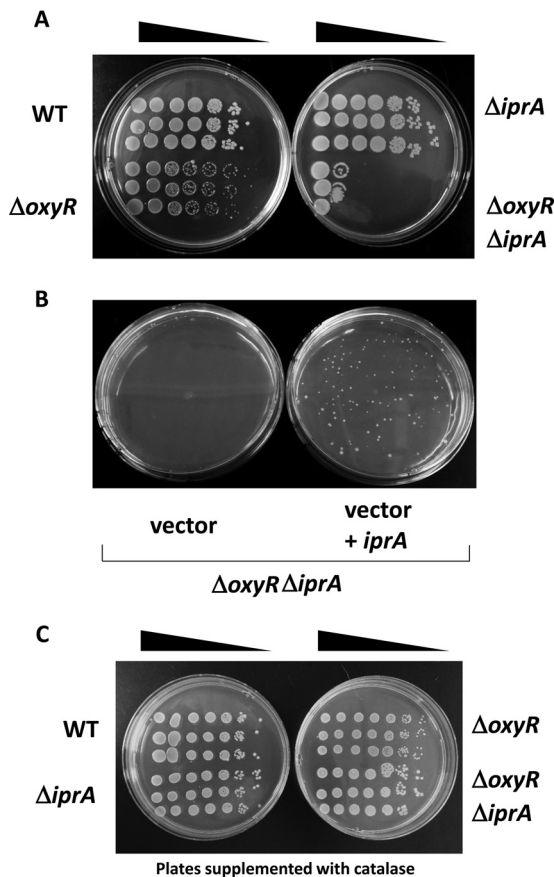


FIG 7 An *S. Typhimurium* $\Delta oxyR \Delta iprA$ mutant strain displays a growth defect on solid medium. (A) *S. Typhimurium* $\chi 3339$ WT, $\Delta oxyR$ mutant, $\Delta iprA$ mutant, and $\Delta oxyR \Delta iprA$ mutant strains were grown in liquid medium to equivalent OD₆₀₀ values, serially diluted, and plated on solid medium for colony growth. (B) Strain $\chi 3339 \Delta oxyR \Delta iprA$ containing either vector or vector plus *iprA* was diluted and plated for single-colony growth on solid medium. (C) The solid medium was supplemented with catalase protein (140 μ g per ml of medium), and the indicated strains were grown and plated as described for panel A.

RNA-Seq analysis of *S. Typhimurium* $\Delta iprA$ mutant. It is possible that any phenotypes observed in a $\Delta iprA$ mutant (including the increase in oxidative stress resistance) are the result of changes in gene expression due to loss of the *iprA* gene. To identify genes altered in expression in the *S. Typhimurium* $\Delta iprA$ mutant, we performed RNA-Seq analysis using total RNA obtained from *S. Typhimurium* $\chi 3339 \Delta iprA$ compared to the isogenic WT strain in both log and stationary phase. Quality control parameters from the analysis are available in Table S2 in the supplemental material. We identified 82 and 56 genes that were altered in expression in the $\Delta iprA$ mutant compared to the WT in log and stationary phases, respectively (Table 2; see also the supplemental material for full data set). Many of these hits were found to reside in the same operon or functional gene set, and several hits were observed in both phases of growth (Table 2). We used RT-qPCR to successfully confirm gene hits from the RNA-Seq analysis, and we used complementation via a plasmid copy of the *iprA* gene to restore WT gene expression phenotypes in the $\Delta iprA$ mutant strain (Fig. 8A).

Genes involved in type I fimbria formation, including *fimA*, *fimW*, *fimI*, *fimC*, *fimD*, *fimH*, *fimZ*, and *fimW*, were found to be

decreased in expression in the $\Delta iprA$ mutant in either log or stationary phase, or both (Table 2). We also found other fimbrial genes (*stcB*, SL1344_2934, *stfE*, *stbA*, *stbB*, and *stdA*) to be altered in expression as well in the $\Delta iprA$ mutant strain (Table 2). The PTS transport system (found in genes SL1344_4466 through SL1344_4471) is increased in expression in the $\Delta iprA$ mutant in log phase (with a single gene hit from this system, SL1344_4468, being decreased in expression in stationary phase) (Table 2). In log phase, the plasmid-borne *spvR* and *spvABCD* genes involved in *S. Typhimurium* virulence were found to be increased in expression in the $\Delta iprA$ mutant (Table 2). In stationary phase, the large ethanolamine utilization gene operon (genes SL1344_2425 to SL1344_2433) was increased in expression in the $\Delta iprA$ mutant strain (Table 2). Many genes associated with phage were altered in expression in the $\Delta iprA$ mutant (SL1344_0949, SL1344_1932, SL1344_1959 to SL1344_1963, SL1344_2213, SL1344_2680, SL1344_2681 to SL1344_2683, SL1344_1703, SL1344_4136 to SL1344_4140, SL1344_4141 to SL1344_4153, and SL1344_4154) (Table 2). A large number of genes (48 [34% of the total hits]) encoding hypothetical or putative proteins were altered in expression in the $\Delta iprA$ mutant, including operons SL1344_1488 to SL1344_1491, SL1344_1659 to SL1344_1661, and SL1344_0637 and SL1344_0638 (Table 2).

Among the strongly altered genes (those with >5-fold change in expression) in both log and stationary phase were *fliC*, *fljA*, *fljB* (aka *flaG*), and *hin*, involved in the *S. Typhimurium* flagellin phase switch (between flagellin type 2 and flagellin type 1). The WT background strain $\chi 3339$ expresses flagellin type 2 (FljB/FlaG), and the RNA-Seq data indicated a phase switch to flagellin type 1 (FljC) in the $\Delta iprA$ mutant (Table 2). This phase switch was confirmed using protein gel analysis (data not shown) and chromosomal DNA PCR analysis (Fig. 8B), indicating correspondence to RNA-Seq data and that the phase switch occurred via the “textbook” mechanism of Hin-mediated DNA inversion at the *fljAB* promoter and not via some other uncharacterized mechanism associated with the $\Delta iprA$ mutation. However, when we attempted to complement this phenotype (back to the flagellin type 2 phenotype) using a plasmid-borne copy of the WT *iprA* gene, the complementation did not occur (data not shown). We then screened a panel of $\chi 3339$ background strains in our lab containing a range of different mutations, and we found a mixture of flagellin phase types across these strains, indicating that the phase switch appeared to occur randomly (data not shown). Thus, the flagellin phase switch observed in the $\Delta iprA$ mutant is likely a manifestation of stochastic events, although it is worth noting that the specific signals that regulate the timing or frequency of phase switch mechanisms are currently not well characterized (44).

We did not observe changes in the expression of previously characterized oxidative stress genes in the $\Delta iprA$ mutant within the cutoff values of our RNA-Seq analysis. However, the expression of the *katE* gene was increased 1.92-fold (just below the cutoff of 2-fold) in the $\Delta iprA$ mutant in log phase, with a statistically significant *P* value (data not shown). This is consistent with the phenotype of increased oxidative stress resistance in the *S. Typhimurium* $\Delta iprA$ mutant and its partial dependence on the *katE* gene, and it suggests that an increase in *katE* gene expression might play a role in the $\Delta iprA$ mutant phenotype (although the different aspects of this phenomenon are discussed below). The other genes analyzed in Fig. 6 (*katG*, *katN*, *rpoS*, *dps*, *ahpC*, and

TABLE 2 Genes altered in expression in *Salmonella* Typhimurium χ 3339 Δ *iprA*

Growth phase and gene	Gene name and/or product	Fold difference ^a	Up- or downregulation	P value
Log phase				
Metabolism genes				
SL1344_0068	<i>carB</i> ; carbamoyl-PO ₄ synthase	2.42	Up	<1.0E-300
SL1344_0114	<i>leuL</i> ; <i>leu</i> operon leader	2.9	Down	0.0492303
SL1344_0355	Terminal oxidase subunit	2.12	Up	2.38E-146
SL1344_0456	<i>glmK</i> ; nitrogen regulatory protein	2.9	Down	0.0492303
SL1344_0548	Bactoprenol glucose transferase	2.74	Down	1.25E-09
SL1344_0549	Bactoprenol glucose transferase	3.09	Down	7.95E-05
SL1344_0743	<i>oadG2</i> ; oxaloacetate decarboxylase	2.76	Down	0.0215319
SL1344_1238	<i>argD</i> ; acetylornithine aminotransferase	2.09	Up	2.29E-49
SL1344_1488	<i>glgX</i> ; glycogen protein	2.08	Up	8.93E-288
SL1344_2017	<i>pduD</i> ; diol dehydratase	2.01	Up	3.53E-27
SL1344_2081	<i>cpsG</i> ; phosphomannomutase	3.43	Up	1.19E-08
SL1344_2900	Decarboxylase	2.48	Up	0.0105352
SL1344_4390	<i>pyrI</i> ; aspartate carbamoyltransferase	4.95	Up	<1.0E-300
SL1344_4391	<i>pyrB</i> ; aspartate carbamoyltransferase	4.12	Up	<1.0E-300
PTS transport system genes				
SL1344_4466	PTS transporter	3.15	Up	1.31E-16
SL1344_4467	PTS transporter	2.82	Up	5.13E-17
SL1344_4468	PTS transporter	3.06	Up	5.59E-36
SL1344_4469	PTS transporter	2.38	Up	3.84E-45
SL1344_4470	Putative sugar isomerase	2.49	Up	2.50E-57
SL1344_4471	Putative sugar isomerase	2.14	Up	5.10E-58
Fimbrial genes				
SL1344_0536	<i>fimA</i> ; type 1 fimbrial protein	2.24	Down	2.62E-253
SL1344_0545	<i>fimW</i> ; fimbrial protein	2.11	Down	3.63E-15
SL1344_2128	<i>stcB</i> ; fimbrial chaperone	2.8	Down	2.84E-05
SL1344_2934	Putative fimbrial subunit	3.92	Down	0.000383
Virulence genes				
SL1344_1026	<i>pipA</i> ; hypothetical protein	2.33	Down	3.52E-18
P1_0066	<i>spvR</i> ; plasmid virulence	2.13	Up	2.01E-10
P1_0067	<i>spvA</i> ; plasmid virulence	2.31	Up	1.23E-261
P1_0068	<i>spvB</i> ; plasmid virulence	2.84	Up	7.31E-279
P1_0069	<i>spvC</i> ; plasmid virulence	3.6	Up	1.12E-63
P1_0070	<i>spvD</i> ; plasmid virulence	3.74	Up	2.48E-34
Phage genes				
SL1344_0949	Bacteriophage protein	2.52	Down	0.0001826
SL1344_1932	Putative phage protein	2.76	Up	0.0286893
SL1344_1959	Phage protein	2.15	Down	1.99E-07
SL1344_2673	Putative DNA invertase	5.55	Down	8.40E-05
SL1344_2680	Putative phage base protein	3.32	Down	0.0369301
SL1344_2682	Putative phage tail protein	2.23	Down	0.0201203
SL1344_2683	Phage lysis protein	3.22	Down	0.0163595
SL1344_2693	Terminase subunit	2.32	Down	0.0004644
SL1344_2703	Bacteriophage protein	2.21	Down	0.0252955
SL1344_4136	Putative phage protein	2.87	Up	4.28E-05
SL1344_4141	<i>gtrA</i> ; phage glycosyltransferase	2.17	Down	0.000599
SL1344_4153	Endolysin	2.13	Down	0.002344
SL1344_4154	Bacteriophage protein	2.51	Down	0.0062789
Other genes				
SL1344_0044	<i>rpsT</i> ; 30S ribosomal protein S20	2.24	Down	3.15E-253
SL1344_0490	Secreted protein	2.19	Down	1.49E-12
SL1344_0705	ABC transporter protein	2.72	Up	0.0166391
SL1344_1179	<i>envF</i> ; lipoprotein	2.13	Up	0.0152697
SL1344_1422	ABC transporter protein	2.05	Up	1.59E-29
SL1344_1489	Putative hydrolase	2.16	Up	1.11E-02

(Continued on following page)

TABLE 2 (Continued)

Growth phase and gene	Gene name and/or product	Fold difference ^a	Up- or downregulation	P value
SL1344_1490	Putative hydrolase	2.15	Up	2.61E-256
SL1344_1888	<i>fliC</i> ; flagellin 1	5.48	Up	<1.0E-300
SL1344_2755	<i>fljA</i> ; repressor of flagellin 2	143.1	Down	<1.0E-300
SL1344_2756	<i>flaG</i> ; flagellin 2	16.88	Down	<1.0E-300
SL1344_2757	<i>hin</i> ; DNA invertase	6.08	Down	<1.0E-300
SL1344_2943	<i>ycgZ</i> ; glucarate transporter	2.08	Down	1.14E-81
SL1344_3183	<i>rpsU</i> ; 30S ribosomal protein S21	2.04	Down	3.55E-213
SL1344_3807	<i>rnpA</i> ; RNase P component	2.14	Down	3.43E-68
SL1344_4052	Inner membrane protein	2.59	Down	0.0034985
Genes encoding hypothetical/putative proteins				
SL1344_0291	Hypothetical protein	2.58	Down	0.0001506
SL1344_0354	Hypothetical protein	2.39	Up	2.29E-42
SL1344_0544	Hypothetical protein	2.56	Down	8.51E-05
SL1344_0575	Hypothetical protein	4.55	Up	0.0470614
SL1344_0736	Hypothetical protein	2.21	Up	<1.0E-300
SL1344_0815	Hypothetical protein	2.42	Down	0.0323411
SL1344_1059	Hypothetical protein	2.57	Up	<1.0E-300
SL1344_1297	Hypothetical protein	2.29	Down	1.75E-05
SL1344_1443	Hypothetical protein	2.47	Up	8.92E-65
SL1344_1491	Hypothetical protein	2.04	Up	4.57E-44
SL1344_1516	Putative lipoprotein	2.15	Down	0.0099574
SL1344_1519	Putative regulatory protein	2.09	Up	5.01E-36
SL1344_1659	Hypothetical protein	3.36	Up	1.11E-02
SL1344_1660	Hypothetical protein	3.37	Up	1.83E-234
SL1344_1661	Hypothetical protein	4.67	Up	4.52E-205
SL1344_1830	Pseudogene	2.25	Down	0.0003631
SL1344_2116	Hypothetical protein	2.62	Down	0.0050997
SL1344_2185	Hypothetical protein	2.17	Down	9.98E-05
SL1344_2343	Putative DNA-binding protein	4.53	Down	0.0043958
SL1344_2345	Putative lipoprotein	2.67	Down	0.0002584
SL1344_2887	Hypothetical protein	2.94	Down	3.65E-10
SL1344_3653	Hypothetical protein	2.08	Up	3.17E-42
SL1344_3910	Hypothetical protein	2.1	Down	0.000527
SL1344_4257	Hypothetical protein	3.02	Down	5.05E-06
Stationary phase				
Metabolism genes				
SL1344_0743	<i>oadG2</i> ; oxaloacetate decarboxylase	3.05	Down	0.001348
Ethanolamine utilization genes				
SL1344_2425	<i>eutJ</i> ; ethanolamine utilization	2.46	Up	1.21E-18
SL1344_2426	<i>eutE</i> ; aldehyde dehydrogenase	2.24	Up	4.00E-35
SL1344_2427	<i>eutN</i> ; ethanolamine utilization	2.35	Up	9.54E-10
SL1344_2429	<i>eutD</i> ; phosphate acyltransferase	2.22	Up	3.40E-11
SL1344_2430	Cobalamin adenosyltransferase	2.17	Up	1.27E-09
SL1344_2431	<i>eutQ</i> ; ethanolamine utilization	2.44	Up	7.97E-18
SL1344_2432	<i>eutP</i> ; ethanolamine utilization	3.7	Up	1.02E-16
SL1344_2433	<i>eutS</i> ; ethanolamine utilization	2.13	Up	0.0076109
Fimbrial genes				
SL1344_0199	<i>stfE</i> ; fimbrial subunit StfE	2.12	Down	0.0395983
SL1344_0334	<i>stbB</i> ; fimbrial chaperone	5.08	Down	0.0214963
SL1344_0335	<i>stbA</i> ; fimbrial protein	3.54	Up	0.010675
SL1344_0536	<i>fimA</i> ; type 1 fimbrial protein	4.77	Down	<1.0E-300
SL1344_0537	<i>fimI</i> ; pilin protein	3.84	Down	3.81E-46
SL1344_0538	<i>fimC</i> ; fimbrial chaperone	3.6	Down	3.04E-33
SL1344_0539	<i>fimD</i> ; usher protein	2.64	Down	3.44E-36
SL1344_0540	<i>fimH</i> ; fimbrial protein	2.56	Down	6.68E-20
SL1344_0542	<i>fimZ</i> ; transcription regulator	3.59	Down	4.10E-10

(Continued on following page)

TABLE 2 (Continued)

Growth phase and gene	Gene name and/or product	Fold difference ^a	Up- or downregulation	P value
SL1344_0545	<i>fimW</i> ; fimbrial protein	2.18	Down	2.66E-07
SL1344_2934	Putative fimbrial subunit	7.11	Down	0.002188
SL1344_3008	<i>stdA</i> ; fimbrial protein	3.37	Down	1.39E-13
Phage genes				
SL1344_1963	Bacteriophage protein	2.2	Down	6.97E-05
SL1344_2213	Bacteriophage holin	2.58	Up	0.0242426
SL1344_4140	<i>gtrB</i> ; phage glycosyltransferase	2.31	Down	6.40E-06
Other genes				
SL1344_0044	<i>rpsT</i> ; 30S ribosomal protein S20	2.14	Down	6.00E-177
SL1344_0275	<i>sciN</i> ; lipoprotein	8.86	Up	0.0004054
SL1344_0278	<i>sciQ</i> ; putative membrane protein	2.67	Up	0.0290844
SL1344_0490	Secreted protein	3.87	Down	1.28E-07
SL1344_0577	<i>fepE</i> ; ferric enterobactin	2.28	Down	2.55E-16
SL1344_0583	<i>entC</i> ; isochorismate synthase	2.12	Down	1.62E-05
SL1344_0705	ABC transporter protein	2.59	Down	0.0060809
SL1344_1285	Ferredoxin-like protein	2.23	Up	0.009504
SL1344_2755	<i>fljA</i> ; repressor of flagellin 2	18.72	Down	3.31E-119
SL1344_2756	<i>flaG</i> ; flagellin 2	17.92	Down	<1.0E-300
SL1344_1888	<i>fliC</i> ; flagellin 1	3.62	Up	<1.0E-300
SL1344_3144	Membrane transport protein	2.16	Down	0.0002762
SL1344_4052	Inner membrane protein	3.22	Down	0.0088688
SL1344_4468	PTS transporter	2.14	Down	9.37E-05
Genes encoding hypothetical/putative proteins				
SL1344_0011	Hypothetical protein	3.92	Down	0.0004655
SL1344_0017	Hypothetical protein	2.71	Down	0.0334128
SL1344_0032	Hypothetical protein	7.11	Down	0.002188
SL1344_0100	Hypothetical protein	2.31	Down	0.0192471
SL1344_0544	Hypothetical protein	4.32	Down	0.0040759
SL1344_0637	Putative hydrolase	13.78	Up	0.0009813
SL1344_0638	Putative hydrolase	2.45	Up	1.19E-05
SL1344_0702	Putative glycosyltransferase	5.08	Down	0.0214963
SL1344_0706	Putative glycosyltransferase	16.76	Down	1.57E-08
SL1344_0785	Putative inner membrane protein	6.6	Down	2.60E-07
SL1344_0814	Hypothetical protein	3.05	Down	0.0004317
SL1344_0815	Hypothetical protein	3.2	Up	0.0491571
SL1344_1516	Putative lipoprotein	2.27	Down	0.0131079
SL1344_2185	Hypothetical protein	3.16	Down	6.42E-06
SL1344_2343	Putative DNA-binding protein	3.05	Down	0.0139479
SL1344_2379	Hypothetical protein	2.12	Down	1.93E-20
SL1344_2738	Putative hexulose-6-phosphate synthase	2.31	Down	2.46E-11
SL1344_4402	Hypothetical protein	3.81	Down	2.06E-07

^a Fold difference is the fold amount that gene expression is up- or downregulated in the $\Delta iprA$ mutant compared to the WT.

tssA) did not display an expression change of >1.5-fold difference in the $\Delta iprA$ mutant compared to the WT (data not shown).

DISCUSSION

Our work has found that the protein encoded by the bacterial *iprA* gene is highly conserved, and that deletion of the *iprA* gene results in a dramatic increase in resistance to oxidative stress. How does the IprA protein function in relation to its role in oxidative stress resistance? A useful approach for framing such a discussion is to ask whether IprA is working at the transcriptional level (controlling transcription of relevant genes), at the posttranscriptional level (the IprA protein has some sort of enzymatic or protein in-

teraction activity related to oxidative stress resistance), or at both levels. To address this question (and since the *iprA* gene was previously uncharacterized), we performed RNA-Seq analysis comparing the *S. Typhimurium* $\Delta iprA$ mutant and WT strains. We did not observe changes in the expression of previously characterized oxidative stress resistance genes beyond the 2-fold cutoff level of the analysis, but we did measure an increase in *katE* gene expression at a 1.92-fold greater level in the $\Delta iprA$ mutant strain than in the WT. This is consistent with the role of *katE* observed in the $\Delta iprA$ mutant phenotype but is likely not the whole story, since the effect of the *katE* gene was partial, and the *rpoS* gene appears to control other functions involved in the $\Delta iprA$ mutant phenotype

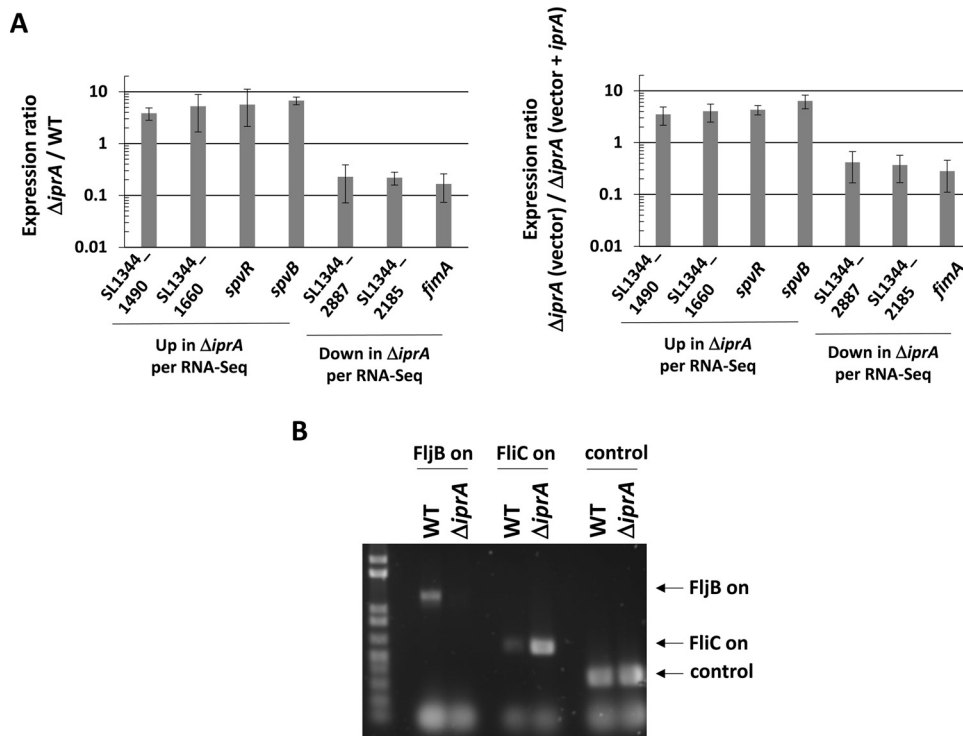


FIG 8 Confirmation of RNA-Seq results. (A) RT-qPCR analysis was performed using total RNA harvested from *S. Typhimurium* χ 3339 WT and $\Delta iprA$ mutant strains (graph on left) and from χ 3339 $\Delta iprA$ containing either vector or vector plus *iprA* (graph on right). The qPCR was performed using primers hybridizing to the indicated genes identified from the RNA-Seq results shown in Table 2. The qPCR product levels were normalized to the level of the *ipxC* gene, and a ratio of each gene level for the $\Delta iprA$ mutant to the WT (left) or the $\Delta iprA$ mutant (vector) to the $\Delta iprA$ mutant (vector plus *iprA*) (right) was calculated to give a fold difference in expression between the corresponding samples. Differences in expression between the samples for each gene were significant at a *P* value of <0.05 , using a *t* test to compare WT and $\Delta iprA$ mutant samples. (B) DNA analysis of flagellin phase switch in *S. Typhimurium* χ 3339 $\Delta iprA$ compared to the WT. PCR products obtained from chromosomal DNA were analyzed to confirm the flagellin phase switch indicated by the RNA-Seq data. Primers that amplify PCR products indicative of either the FljB on (flagellin type 2) or FliC on (flagellin type 1) orientation of the *fljA-fljB-hin* locus were used to amplify the corresponding DNA fragments from the indicated strains, and the products were run on an agarose gel and stained for fluorescence. In addition, a control product not affected by phase switch was also amplified and run on the gel. The results of the DNA analysis confirm the switch to FliC on in the $\Delta iprA$ mutant strain.

beyond *katE*. Thus, the activity of IprA in relation to oxidative stress resistance appears to involve both KatE and another RpoS-controlled factor. IprA activity might involve protein-protein interactions between IprA and these factors that act at the posttranscriptional level to lower oxidative stress resistance. It is also possible that IprA acts at both the transcriptional level (i.e., is involved in transcriptional control pathways) and the posttranscriptional level (altering protein activity). Moreover, it is possible that some of the genes identified as altered in expression in the $\Delta iprA$ mutant are involved in oxidative stress resistance but have not been previously characterized as such. Another possibility is that the IprA protein is a target for reactive oxygen species in the cell, such that the interaction of the oxygen species with IprA is lethally toxic to the cells, and the removal of IprA results in increased survival in oxidative stress (see below for further discussion).

In our RNA-Seq analysis, we found that the deletion of *S. Typhimurium iprA* resulted in the altered expression of 82 and 56 genes in log and stationary phase, respectively, and these results were verified using RT-qPCR and complementation analysis. This indicates that IprA is involved in regulation pathways that control gene expression. We currently do not know if IprA participates in gene regulation via binding to DNA directly, via interactions with RNA, or via interactions with proteins that serve to regulate downstream gene expression or signaling pathways. However, the IprA protein contains a pre-

dicted winged helix-turn-helix (wHTH) DNA-binding domain in the C terminus, and deletion of the C-terminal 10 amino acids abolished WT IprA activity. Thus, IprA could use the wHTH to bind DNA as part of its action, and future work will be targeted at testing this activity. Based on deletion analysis, the predicted CAP effector binding domain of IprA does not appear to be involved with the role of IprA in oxidative stress resistance.

The genes altered in expression in the *S. Typhimurium* $\Delta iprA$ mutant are distributed across the *S. Typhimurium* genome and belong to varied functional groups, including virulence, fimbria production, PTS transport, ethanolamine utilization, various phage proteins, and many genes encoding hypothetical or putative proteins. We currently do not know if or how these genes potentially participate in the role of IprA in oxidative stress resistance. It is likely that IprA is involved in other functions beyond oxidative stress resistance, and further study based on the gene hits identified here may allow other IprA functions to be discovered. In regard to a possible link between IprA and the *S. Typhimurium* response to rotating-wall-vessel conditions, we found that the deletion of *iprA* had no effect on *S. Typhimurium* phenotypes observed in this environment (data not shown).

We found that the deletion of both *oxyR* and *iprA* resulted in a *S. Typhimurium* strain that displayed a severe growth defect on solid media. This is notable since both the *oxyR* and *iprA* genes

participate in oxidative stress resistance, with OxyR controlling oxidative stress resistance genes and IprA serving to downregulate resistance to oxidative stress. It could be that the removal of both genes in the same strain causes genes/proteins associated with OxyR and IprA to act in an abnormal fashion, such that their additive or synergistic effects cause a growth defect. The effects of OxyR and IprA may act to balance genes/proteins involved in oxidative stress resistance, and when both OxyR and IprA are absent, this balance is abolished, and defective gene expression and/or protein activity cause growth problems. We found that supplementation of the agar medium with catalase protein served to reverse the phenotype, which means that the $\Delta oxyR \Delta iprA$ mutant strain is hypersensitive to enhanced oxidative stress on solid medium. This is an interesting observation since the $\Delta iprA$ mutant causes increased oxidative stress resistance in broth, while the $\Delta iprA$ mutation in the presence of the $\Delta oxyR$ mutation causes decreased resistance on plates (suggesting additive effects or interaction of the two resistance schemes under certain conditions). Further study is needed to understand the nature of this phenomenon, but this observation serves as another piece of evidence of the involvement of IprA in the oxidative stress resistance program in bacteria.

Why would evolution select for a bacterial gene that serves to dramatically decrease resistance to oxidative stress resistance? This suggests that insufficient negative regulation of oxidative stress resistance is detrimental to fitness, possibly due to the use of excess metabolic energy in the expression of these functions. In addition, it is established that certain oxidative stress resistance mechanisms can be potentially mutagenic (45–48). Therefore, the evolution of regulation schemes to inhibit these mechanisms would have served to keep resulting mutations “in check,” which would likely be beneficial in the long term. Another possibility is that for some reason, the IprA protein itself is a target for oxidative stress, such that when hit with oxidative damage, it is highly harmful or toxic to the cell (and deletion of the gene removes this target). Future work will be directed at further understanding the selective benefits of the strong downregulation of oxidative stress resistance involving IprA.

Finally, this work serves to highlight the pool of currently uncharacterized genes that are conserved across bacterial genomes and that play as-of-yet-undiscovered roles in bacterial biology. These genes represent an “untapped pool” of potentially useful genes that might play a role in bacterial engineering, vaccine strain design, and other applications. Many of these untapped genes are highly conserved across different bacteria, which strongly suggests evolutionary selection for important functions. In addition, learning the functions of these uncharacterized yet highly conserved genes will allow greater understanding of bacterial growth, survival, and regulation across genera, including those containing pathogenic species.

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