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New Role for the *ibeA* Gene in H_2O_2 Stress Resistance of *Escherichia coli*

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***ibeA* is a virulence factor found in some extraintestinal pathogenic *Escherichia coli* (ExPEC) strains from the B2 phylogenetic group and particularly in newborn meningitic and avian pathogenic strains. It was shown to be involved in the invasion process of the newborn meningitic strain RS218. In a previous work, we showed that in the avian pathogenic *E. coli* (APEC) strain BEN2908, isolated from a colibacillosis case, *ibeA* was rather involved in adhesion to eukaryotic cells by modulating type 1 fimbria synthesis (M. A. Cortes et al., Infect. Immun. 76:4129–4136, 2008). In this study, we demonstrate a new role for *ibeA* in oxidative stress resistance. We showed that an *ibeA* mutant of *E. coli* BEN2908 was more sensitive than its wild-type counterpart to H_2O_2 killing. This phenotype was also observed in a mutant deleted for the whole GimA genomic region carrying *ibeA* and might be linked to alterations in the expression of a subset of genes involved in the oxidative stress response. We also showed that RpoS expression was not altered by the *ibeA* deletion. Moreover, the transfer of an *ibeA*-expressing plasmid into an *E. coli* K-12 strain, expressing or not expressing type 1 fimbriae, rendered it more resistant to an H_2O_2 challenge. Altogether, these results show that *ibeA* by itself is able to confer increased H_2O_2 resistance to *E. coli*. This feature could partly explain the role played by *ibeA* in the virulence of pathogenic strains.**

Escherichia coli is a bacterial species found mainly in the gut of humans and warm-blooded animals (53). Besides commensals, a number of strains are responsible for intestinal or extraintestinal infections, the former being grouped under the acronym IPEC (for intestinal pathogenic *Escherichia coli*) and the latter by the acronym ExPEC (for extraintestinal pathogenic *Escherichia coli*) (31). In humans, ExPEC isolates are mainly isolated from cases of urinary tract infections, neonatal meningitis, and septicemia (49). *E. coli* strains isolated from avian species (called APEC, for avian pathogenic *E. coli*) are closely related to strains from the ExPEC group, in terms of both phylogeny and virulence gene profiles (18, 40, 48).

Such a diversity is due in part to the plasticity of the *E. coli* genome, which allows one to distinguish between a core genome that is present in all *E. coli* strains and a set of accessory genes that confer specific properties and that are found in only a fraction of *E. coli* strains (38, 54).

Some of these accessory genes provide the bacteria with specific properties that play roles in the different steps of the infectious process. Concerning APEC strains, studies have identified a few genes that are required for full virulence of the bacteria. Among these are the aerobactin iron capture system, the *tsh* gene, encoding a temperature-sensitive hemagglutinin, the phosphate transport (*pst*) system, and the vacuolating toxin-encoding gene *vat* (14, 15, 33, 45). Our laboratory has been searching for new virulence genes using different screening strategies (21, 47, 50). Based on the observation that APEC strains shared many properties with neonatal meningitis *E. coli* (NMEC) strains and on the identification of *ibeA* as a virulence gene of the neonatal meningitis strain RS218, we investigated the role of *ibeA* in the virulence of APEC (20, 27). In the prototypical neonatal meningitis *E. coli* strain RS218, inactivating *ibeA* caused a significant decrease in the invasion of brain microvascular endothelial cells and decreased its ability to cause meningitis (27). In the APEC strain BEN2908, the deletion of *ibeA* led to a significant reduction of virulence (20). So far, the precise function of IbeA has remained elusive and controversial, as some authors suggest it could be an adhesin, while our studies have shown no such role in APEC strain BEN2908 (9, 59, 60).

In a previous study, we showed that *ibeA* was indeed involved in adhesion of strain BEN2908 to eukaryotic cells but only indirectly via the modulation of the synthesis of type 1 fimbriae (9). In fact, an *ibeA* mutant is less adhesive to human brain microvascular endothelial cells (HBMECs) than the wild-type BEN2908, and this feature is correlated with a decrease in type 1 fimbria expression (9). However, this observation is unlikely to explain the decrease in virulence for chickens of an *ibeA* mutant, as a derivative of strain BEN2908 lacking the entire type 1 fimbria operon is still virulent (39). We therefore concluded that the decreased expression of type 1 fimbriae could not solely explain the decreased virulence of the *ibeA* mutant. A correlate is that IbeA protein must be involved in other cellular processes that take part in bacterial virulence.

We previously observed that IbeA contains a putative FAD binding domain (E value, 6.8 e−06; http://pfam.sanger.ac.uk/) and is predicted to be located in the cytoplasmic fraction of *E. coli* (9). A more recent search indicated that IbeA belongs to the Pfam12831 protein family, which are annotated as FAD-dependent oxidoreductases (E value, 3.06 e−83; http://www.ncbi.nlm.nih.gov). Moreover, IbeA is annotated as a putative dihydrolipoamide dehydrogenase, a class of enzymes that are involved in oxidoreduction reactions. For instance, the *E. coli* dihydrolipoamide dehydrogenase is an oxidoreductase that participates in electron transfer reactions within three multiproteic enzyme complexes: pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase,

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and the glycine cleavage system (Ecocyc.org). In addition, IbeA shares similarities with other oxidoreductases belonging to the FixC family, similarities that extend over the FAD binding domain into the first 150 amino acids of IbeA. Genes encoding proteins belonging to the FixC family have been studied in several nitrogen-fixing bacteria. Their exact functions still remain to be elucidated, but they are proposed to be involved in oxidoreductive reactions. For example, in *Rhodospirillum rubrum*, a FixC homologue would belong to a complex involved in electron transfer to nitrogenase, and a *fixC* mutant of this bacterium presents metabolic alterations reflecting an imbalance in its redox status (16). These data suggest that IbeA is involved in some sort of oxidoreductive reaction or regulation of the cellular redox status.

In this work, we further characterized IbeA by determining its subcellular localization and by investigating its contribution to oxidative stress resistance. Our results led us to suggest that IbeA is involved in H_2O_2 resistance in *E. coli*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids are described in Table 1. *E. coli* strains were grown in LB-Lennox medium (35) or in M9 medium supplemented with 10 mM glucose as the carbon source and with trace salts (15 mg · liter^{-1} $Na_2EDTA \cdot 2H_2O$, 4.5 mg · liter^{-1} $ZnSO_4 \cdot 7H_2O$, 300 μg · liter^{-1} $CoCl_2 \cdot 6H_2O$, 1 mg · liter^{-1} $MnCl_2 \cdot 4H_2O$, 1 mg · liter^{-1} H_2O, 1 mg · liter^{-1} H_3BO_3, 400 μg · liter^{-1} $Na_2MoO_4 \cdot 2H_2O$, 3 mg · liter^{-1} $FeSO_4 \cdot 7H_2O$, 300 μg · liter^{-1} $CuSO_4 \cdot 5H_2O$) and thiamine (0.1 g · liter^{-1}) (M9sup medium).

Cells were first grown overnight in LB medium, harvested, and washed twice in M9sup medium before concentration to an optical density at 600 nm (OD_{600}) of 3. Cells were then inoculated in M9sup medium at a 60-fold dilution. *E. coli* strains were grown aerobically at 37°C; growth was monitored by determining the OD_{600}. Ampicillin (100 μg · ml^{-1} for *E. coli* BEN2908 derivatives and 50 μg · ml^{-1} for other *E. coli* strains), kanamycin (50 μg · ml^{-1}), and nalidixic acid (30 μg · ml^{-1}) were used when necessary.

DNA techniques and strain constructions. Restriction endonucleases (New England BioLabs) were used according to the manufacturer's instructions. DNA fragments were purified from agarose gels by use of the Nucleospin Extract II purification kit (Macherey-Nagel).

Primers used in this study are described in Table 1. PCRs were performed with an Applied Biosystems model 9700 apparatus, using 1 U *Taq* DNA polymerase from New England BioLabs in 1× buffer, a 200 μM concentration of each deoxynucleoside triphosphate, 0.8 μM each primer, and 10 ng of chromosomal DNA in a 50-μl reaction volume. Cycling conditions were 1 cycle of 5 min at 95°C; 30 cycles of 10 s at 95°C, 10 s at 52°C, and 1 min/kb at 72°C; and a final extension of 5 min at 72°C. PCR products were separated in 1% agarose gels for 1 h at 10 V/cm of gel.

A derivative of strain BEN2908 carrying a C-terminal hemagglutinin (HA)-tagged *ibeA* gene was obtained using pSU315 and the method described by Uzzau et al. with primers MC1 and MC2 (56). From the strain obtained, the HA-tagged *ibeA* gene was then amplified using primers MC3 and MC5, and the PCR fragment was digested using EcoRI and BamHI and subcloned in pHSG575 cut with the same enzymes.

Deletion of the GimA genomic region was obtained as described by Datsenko and Wanner (10) using primers MF93 and MF94. The replacement of GimA by the Kan^r cassette was confirmed by PCR using primers MC52 and PG328. The Kan^r cassette was then removed using plasmid pCP20. The deletion of GimA was confirmed by PCR using primers MC52 and MC63.

Subcellular localization of IbeA protein. Cells were grown in LB medium to an OD_{600} of 0.5. The periplasmic fraction was collected as described by Nossal and Heppel (41): bacteria from 1 ml of culture were harvested by centrifugation and resuspended in 30 μl of TSE buffer (10 mM Tris, pH 8.2, 5 mM EDTA, 20% sucrose) (41). The suspension was incubated for 10 min on ice and centrifuged for 10 min at 12,000 × *g*, and pelleted bacteria were quickly resuspended in 30 μl of Thypo buffer (10 mM Tris, pH 8.2, 0.5 mM $MgCl_2$). After 10 min on ice, bacteria were pelleted and the supernatant kept as the periplasmic fraction. Cytoplasmic and membrane fractions were then collected as described in reference 28: after osmotic shock, bacteria were resuspended in 50 μl of lysis buffer (10 mM Tris, 5 mM EDTA, 0.1 mg · ml^{-1} lysozyme), frozen at −80°C for 5 min, and quickly thawed at 37°C for 5 min. Lysed bacteria were then centrifuged, the pellet was kept as the membrane fraction, and the supernatant was kept as the cytoplasmic fraction.

Western blot analysis. Samples were loaded on an SDS-PAGE gel, blotted on a polyvinylidene difluoride (PVDF) membrane, and used for Western blot detection of either IbeA, RpoS, or the control proteins ß-galactosidase (cytoplasmic protein), OmpA (outer membrane protein), SecG (inner membrane protein), and MalE (periplasmic protein). Anti-HA antibody was from Sigma-Aldrich.

RNA extraction and real-time quantitative reverse transcriptase PCR (RT-PCR) analysis. Total RNA was extracted from 500 μl of bacterial liquid culture (OD_{600} of 0.45). Briefly, bacteria were mixed with 1 ml of RNAprotect bacterial reagent (Qiagen) by vortexing for 5 s. After a 5-min incubation at room temperature, the mix of bacteria and RNAprotect bacterial reagent was centrifuged for 10 min at 5,000 × *g*. The supernatant was removed and the pellet was stored for 1 night at −80°C. The next day, total RNA was extracted from the pellet using the RNeasy minikit (Qiagen) according to the manufacturer's recommendations. To avoid any contamination of the extracts by residual genomic DNA, an on-column DNase digestion was performed during RNA purification by using the RNase-free DNase set (Qiagen). The quality of the RNAs was verified by agarose gel electrophoresis, and a Nanodrop device was used for the determination of the ratios of absorbance at 260 and 280 nm and at 260 and 230 nm.

Quantitative RT-PCR was performed as described by Chouikha et al. (7). Briefly, gene-specific reverse transcription of RNAs was performed with Superscript RT III (Invitrogen), using primer PG 199 for *frr* (housekeeping gene), MF67 for *katE*, MF71 for *osmC*, MF77 for *sodC*, MF81 for *yfcG*, MF83 for *yjaA*, MF73 for *pqiA*, MF65 for *iscS*, and MF79 for *sufA*. Samples without RT were concurrently prepared and analyzed for the absence of contaminating genomic DNA. Real-time quantitative PCR (qPCR) analysis was performed in an iCycler system (Bio-Rad) using Absolute quantitative PCR SYBR green mix (ABgene). Four microliters of cDNAs obtained as described above and diluted 10-fold in nuclease-free distilled water was used for qPCR. The PCR program consisted of 35 amplification/quantification cycles of 95°C for 15 s and 60°C for 1 min, with signal acquisition at the end of each cycle. Primers used for qPCR were PG198/PG199 for *frr*, MF67/MF68 for *katE*, MF71/MF72 for *osmC*, MF77/MF78 for *sodC*, MF81/MF82 for *yfcG*, MF83/MF84 for *yjaA*, MF73/MF74 for *pqiA*, MF65/MF66 for *iscS*, and MF79/MF80 for *sufA*. Equation 1 from Pfaffl was used to determine the expression ratios, using *frr* as a housekeeping gene standard (46).

Determination of cytoplasmic redox potential by fluorescence. The plasmid pHOJ124, carrying an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible gene encoding the yellow fluorescent protein $rxYFP^{149_{202}}$, was introduced into strains BEN2908 and BEN2908 Δ*ibeA* (43). Cells then were grown until the mid-exponential growth phase in glucose-containing M9sup medium in the presence of 1 mM IPTG before fluorescence measurements were performed as described previously (43). Briefly, at an OD_{600} of 0.45, 900 μl of the culture was transferred to a prewarmed cuvette (30°C) and fluorescence monitored continuously at 525 nm, with excitation at 505 nm using a Quanta Master spectrofluorometer (PTI, NJ) equipped with a 75-W xenon lamp. After a stable baseline was obtained (denoted F_{init}), the oxidation state of $rxYFP^{149_{202}}$ was determined by reading the fluorescence after successive addition of 50 μl 3.6 mM 4-DPS (F_{ox}; Sigma-Aldrich) and 100 μl 1 M dithiothreitol (F_{red}) to fully oxidize and reduce the protein, respectively. The fraction of oxidized $rxYFP^{149_{202}}$ was then calculated from the expression $1 - (F_{init} - F_{ox})/(F_{red} - F_{ox})$.

H_2O_2 resistance assay. Cells were grown in glucose-containing M9sup medium for 4 h to an OD_{600} of 0.45, corresponding to the mid-exponential

TABLE 1 Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Designation	Relevant characteristic(s) or sequence	Reference or source
E. coli strains			
BEN2908		Avian ExPEC O2:K1:H5 Nal^r	12
BEN2908 Δ*ibeA*			9
BEN2908 ΔGimA			This study
MG1655		F$^-$ λ$^-$ *ilvG* mutant *rfb-50 rph-1*	*E. coli* Genetic Stock Center
MG1655 Δ*fim*		MG1655 Δ*fimAICDFGH::cat*	32
AAEC198A		MG1655 *fimA*::*lacZ*	3
Plasmids			
pUC13		Amp^r, *lacZ*α	26
pUC23A		*ibeA* gene cloned in pUC13	26
pKD4		Kan^r, template plasmid	10
pKD46		Amp^r, λ Red recombinase gene	10
pCP20		Cm^r Amp^r, yeast Flp recombinase gene, FLP	10
pHSG575			51
pSU315		Kan^r, influenza virus HA epitope	56
Primers			
ibeA::HA	MC1	ACGGTACAGGAACGCTTACAGCAAAATGGCGTAAAAGTCTTT TTATCCGTATGATGTTCCTGAT	
	MC2	GACATAAAAACTGGGTTTTTCTTTCATAACTTTATTCCCTGCAT ATGAATATCCTCCTTAG	
ibeA	MC3	TGTCGAATTCAAATTGGTCGTACAACATTA	
	MC5	TAATGGATCCGCAGAACATGGAATTTTGAC	
frr	PG 198	GCGTAGAAGCGTTCAAAACC	
	PG 199	CAAGATCGGACGCCATAATC	
katE	MF67	AAGCGATTGAAGCAGGCGA	
	MF68	CGGATTACGATTGAGCACCA	
osmC	MF71	GCGGGAAGGGAACAGTATCTA	
	MF72	CATCGGCGGTGGTATCAATC	
sodC	MF77	ATCTGAAAGCATTACCTCCCG	
	MF78	TCGCCTTGCCGTCATTATTG	
yfcG	MF81	GAGGCGAGAACTACAGCATTG	
	MF82	CTATCCGAACGCTCATCACC	
yjaA	MF83	CTGGAAATGAATGAGGGCG	
	MF84	TGGATGTGGAACTGGCGATA	
pqiA	MF73	GTGAAACTGATGGCTTACGGC	
	MF74	TACAACAGGAGCACGAACGC	
iscS	MF65	CAGTTTATGACGATGGACGGA	
	MF66	TGGTGATGATGTGCTTGCC	
sufA	MF79	TTTATTGATGGCACGGAAGTCG	
	MF80	TTTCGCCACAGCCACATTCA	
GimA mutant	MF93	TAGGTCACAATTAGTGGGAGGCTCTGATTGCTGCTTTCAAG GCCGGAAGCCATTGTGTAGGCTGGAGCTG	
	MF94	TCAGGCCTTTGCTTCGTTGAAGCGCAGTAAACGGAAACCTGT AGAAGCATATGGTCCATATGAATATCCTCC	
Upstream of GimA	MC52	ACAGTGTTTTATCTTTGGCG	
Downstream of GimA	MC63	ACCGGATGAATACCCGCATG	
Kan^r cassette	PG328	CGGCCACAGTCGATGAATCC	

growth phase. H_2O_2 was added to the growth medium to a final concentration of 25 mM. Aliquots were collected over time and immediately diluted 10 times in M9sup medium supplemented with 10 U · ml^{-1} of bovine liver catalase (Sigma-Aldrich) to ensure the removal of H_2O_2. Survival analysis was performed by plating on LB agar serial dilutions in physiological water containing 10 U · ml^{-1} of bovine liver catalase.

Superoxide anion resistance assay. Cells were grown in glucose-containing M9sup medium in the absence (negative control) or in the presence of 1 or 10 μM intracellular superoxide generator methyl viologen in the growth medium. The growth was monitored by determining the OD_{600}.

Motility assays. Motility of strains BEN2908 and BEN2908 Δ*ibeA* was evaluated as described previously. Briefly, 5 μl of a log-phase culture was spotted onto a soft LB-agar plate (0.25% agar), and the plate was then incubated at 37°C for 5 h.

Statistical analysis. Statistical analysis of H_2O_2 sensitivity and quantification of gene expression were done by applying the Student's *t* test.

RESULTS

The IbeA protein is located in the cytoplasm of strain BEN2908. To confirm bioinformatic analysis, we decided to first investigate

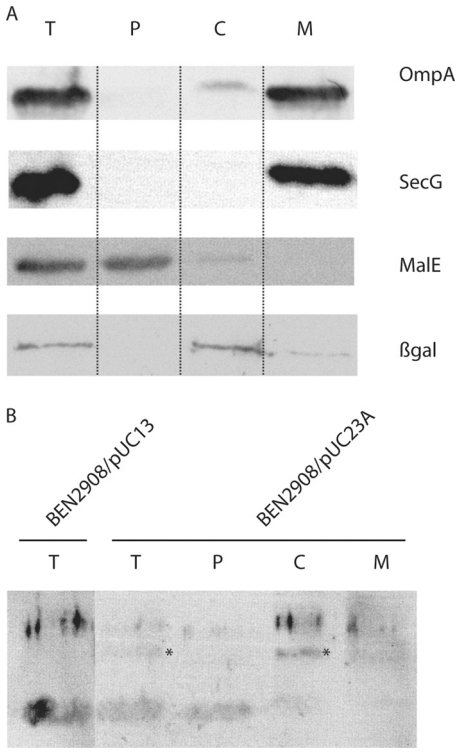

FIG 1 Subcellular localization of the IbeA protein. (A) Subcellular compartments of strain BEN2908/pUC23A were obtained as indicated in Materials and Methods and separated on an SDS-PAGE gel. Control proteins OmpA (outer membrane), SecG (inner membrane), MalE (periplasm), and ß-galactosidase (cytoplasm) were detected to validate the fractionation protocol. T, total cell extract; P, periplasmic fraction; C, cytoplasmic fraction; M, membrane fraction. (B) The IbeA protein was detected by Western blotting using an anti-IbeA antibody. The band corresponding to IbeA is indicated by an asterisk.

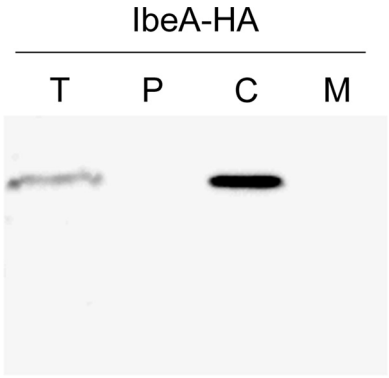

FIG 2 Subcellular localization of the IbeA-HA protein. Subcellular compartments of strain BEN2908/pHSG-*ibeA*::HA were separated as indicated in Materials and Methods and separated on an SDS-PAGE gel. The IbeA-HA protein was detected by Western blotting using an anti-HA antibody. T, total cell extract; P, periplasmic fraction; C, cytoplasmic fraction; M, membrane fraction.

the precise localization of IbeA. Because the expression of IbeA was found to be very low, consistent with the low expression of the *ibeA* gene (7), the analysis was performed using strains transformed with plasmid pUC23A (or pUC13 as a control), which carries *ibeA* and most of the upstream sequence between *ibeR* and *ibeA* (26). It is therefore likely that *ibeA* is expressed under the control of its own promoter. Subcellular compartments corresponding to the cytoplasm, to the periplasm, and to the membranes (both outer and inner) were analyzed by SDS-PAGE followed by Western blotting. Proteins of known localization were used as controls: ß-galactosidase for the cytoplasmic compartment, MalE for the periplasmic fraction, SecG for the inner membrane, and OmpA for the outer membrane. Using the protocol described by Ishidate et al. for *E. coli* K-12 (28), the periplasmic fraction of strain BEN2908 cofractionated with cytoplasmic proteins while control membrane proteins fractionated in the membrane fraction as expected. In this case, IbeA was not recovered into the insoluble fraction containing membrane proteins but was instead collected into the soluble fraction containing both cytoplasmic and periplasmic proteins (data not shown). We then optimized the protocol by including an osmotic shock as a first step to recover periplasmic proteins before lysing bacteria (41). As indicated in Fig. 1A, all control proteins were found in their correct fraction. When these fractions were analyzed with an anti-IbeA antibody, IbeA was found to be located in the cytoplasm (Fig. 1B). Analysis of the culture supernatant indicated that IbeA was not released in the supernatant (data not shown). Because several unspecific bands were obtained with the IbeA antibody, additional experiments were performed. We decided to investigate the localization of an IbeA protein tagged with a C-terminal HA epitope. As indicated in Fig. 2, the IbeA-HA protein was also found in the cytoplasmic fraction. The possibility that the HA tag interfered with a potential membrane localization of IbeA is unlikely, since it has already been shown not to modify the secretion or the localization to the membrane of a number of other proteins (2, 19, 34). Altogether, these results are in agreement with the predicted localization of IbeA in the cytoplasm of *Escherichia coli*.

BEN2908 Δ*ibeA* is more sensitive to H_2O_2 killing. Sensitivity to 25 mM H_2O_2 was analyzed during exponential growth in wild-type BEN2908 and its Δ*ibeA* derivative, which were grown in M9sup-glucose medium. Under these culture conditions, the growth levels of strains BEN2908 and BEN2908 Δ*ibeA* were identical. Prior experiments had indicated that a concentration of 25 mM H_2O_2 was sufficient to kill bacteria but was low enough that the survival could be measured during a 1-h assay (29, 37). While the wild-type strain showed a progressive pattern of killing during H_2O_2 exposure, with a 4-log reduction of CFU after 40 min of exposure and a 4.5-log decrease in survival after 60 min, the Δ*ibeA* mutant presented a much more dramatic phenotype (Fig. 3A). Indeed, from 40 min of H_2O_2 stress onwards, it presented a survival rate significantly lower than that of the wild-type strain, with a 1-log increased mortality. Sixty min after H_2O_2 addition, the survival difference reached 1.5 logs (Fig. 3A). These findings suggested that *ibeA* was involved in H_2O_2 resistance in *E. coli* BEN2908. We also investigated the sensitivity of BEN2908 and its Δ*ibeA* mutant to another kind of oxidative stress, the intracellular generation of superoxide anions by methyl viologen. We monitored the growth of the two strains in M9sup-glucose medium in the absence (negative control) or in the presence of the oxidant. In the presence of 1 μM methyl viologen, the growth rate was slightly decreased in an equivalent manner for the two strains compared to the negative-control conditions. In the presence of a greater dose of methyl viologen (10 μM), the growth was further decreased, but still no difference was observable between BEN2908

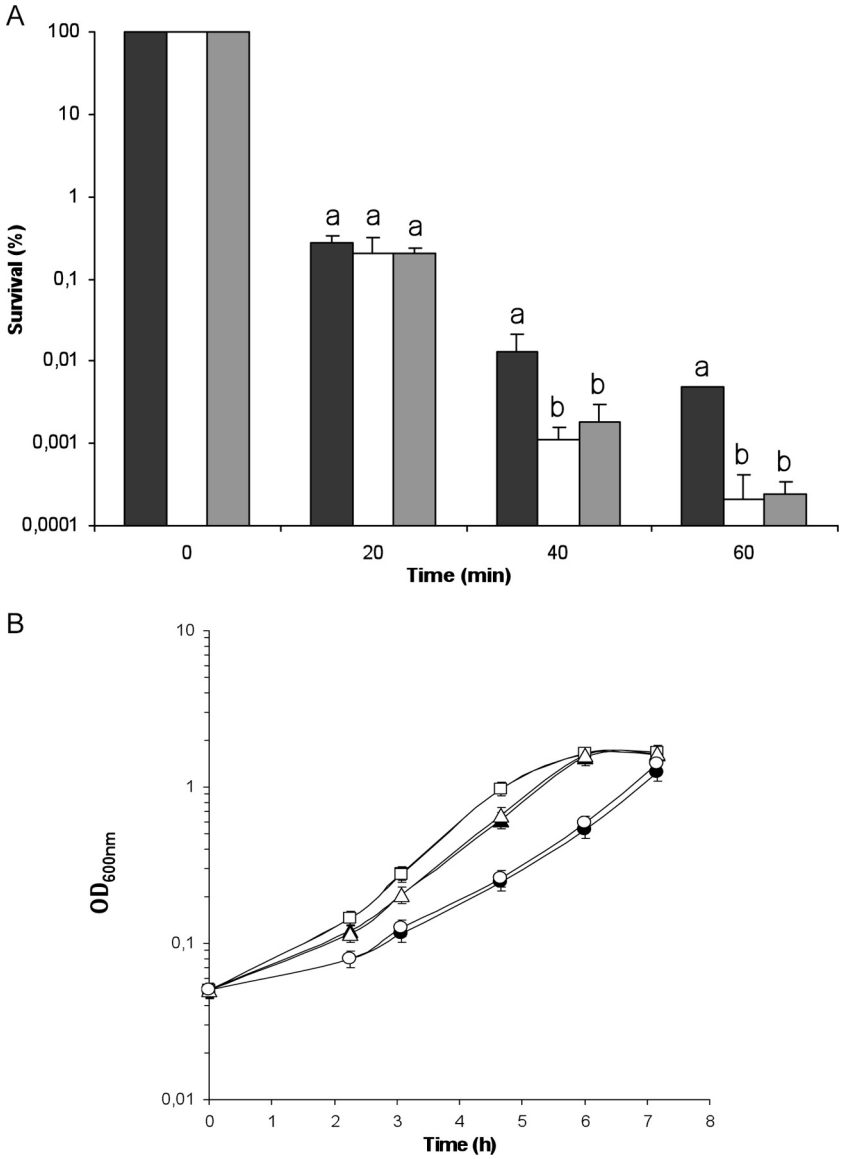

FIG 3 Sensitivity to oxidants of BEN2908 and its Δ*ibeA* and ΔGimA derivatives. (A) The bacteria were grown in glucose-containing M9sup medium until the mid-exponential phase of growth (OD_{600} of 0.45) and were challenged with 25 mM H_2O_2 added directly in the growth medium. The data are represented as percent survival relative to unstressed cells ($t = 0$ min). The results are the means from at least three independent experiments. Error bars show the standard deviations. Black bars, BEN2908 wild type; white bars, BEN2908 Δ*ibeA*; gray bars, BEN2908 ΔGimA. For each time, statistically significant differences in survival rates (Student's *t* test; $P < 0.05$) are indicated by different letters (a and b). (B) The bacteria were grown in glucose-containing M9sup medium in the absence (squares) or in the presence of 1 μM (triangles) or 10 μM (circles) intracellular superoxide generator methyl viologen in the growth medium. The results are the means from at least three independent experiments. Error bars show the standard deviations. Black symbols, BEN2908 WT; white symbols, BEN2908 Δ*ibeA*.

and its Δ*ibeA* mutant. Thus, it seemed that *ibeA* did not intervene in resistance to intracellular superoxide stress (Fig. 3B).

To check that the H_2O_2 sensitivity of the Δ*ibeA* mutant was actually due to the deletion of *ibeA*, we analyzed as described above the H_2O_2 sensitivity of BEN2908 and its Δ*ibeA* mutant complemented either with the pUC23A plasmid carrying ibeA (26) or with the empty vector pUC13. Results indicated that the Δ*ibeA* mutant containing pUC23A presented the same pattern of killing as strain BEN2908 transformed either with pUC23A or pUC13, with no significant difference in survival rates (Fig. 4A). Thus, we concluded that expression of *ibeA* in *trans* from pUC23A restored the survival of strain BEN2908 Δ*ibeA* to a level similar to that of the wild-type strain. Altogether, our results show that the deletion of *ibeA* is actually responsible for the lower resistance to H_2O_2 of the Δ*ibeA* mutant (Fig. 4A).

Some *E. coli* genes involved in the oxidative stress response are downregulated in the Δ*ibeA* mutant. To identify metabolic defaults that could be responsible for the increased sensitivity to H_2O_2 of the Δ*ibeA* mutant, we determined by real-time quantitative RT-PCR analysis the level of transcripts of a range of genes involved in the oxidative stress response in *E. coli* during exponential growth in M9sup-glucose medium: *katE*, *osmC*, *sodC*, *yfcG*, *yjaA*, *pqiA*, *iscS*, and *sufA* (Fig. 5). These genes code for the monofunctional catalase hydroperoxidase II KatE, the osmotically in-

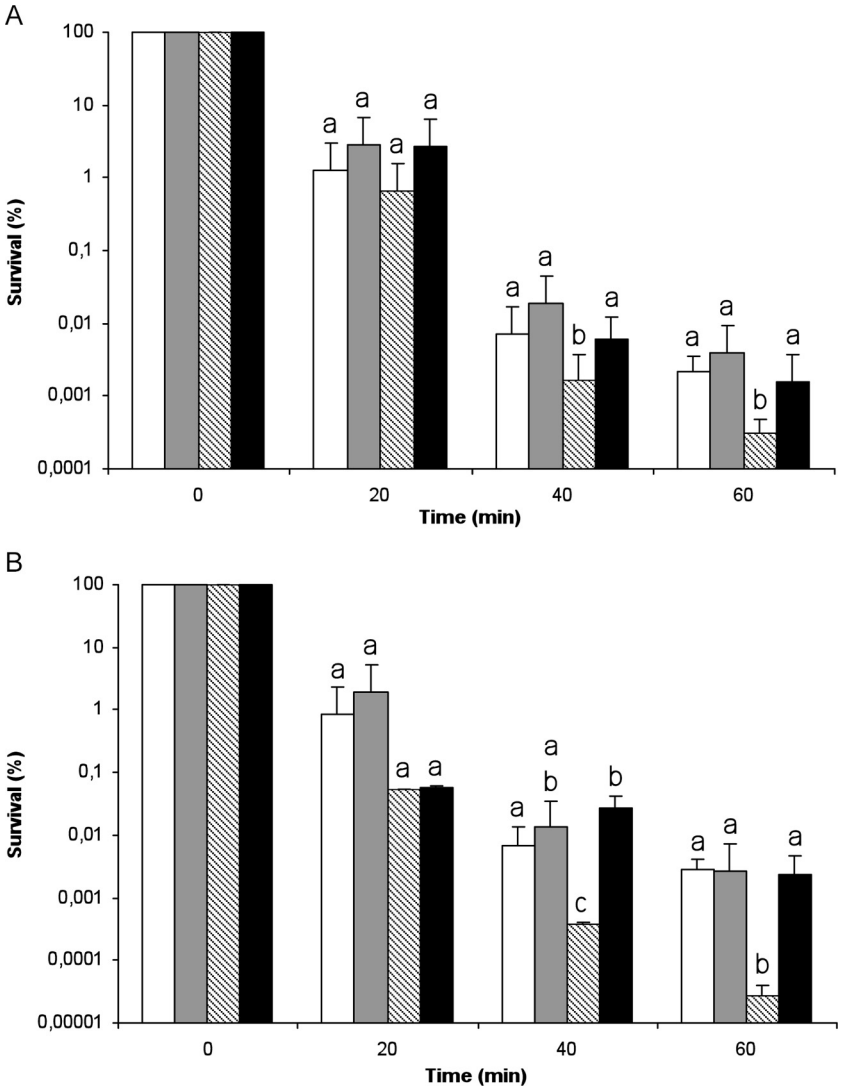

FIG 4 Sensitivity to H_2O_2 killing in strains carrying the pUC23A and pUC13 plasmids. Sensitivity to H_2O_2 of BEN2908 Δ*ibeA* (A) or BEN2908 ΔGimA (B) carrying either the pUC13 vector or the *ibeA*$^+$ pUC23A plasmid was compared to that of the wild-type strain BEN2908 carrying the same plasmids. Bacteria were treated with H_2O_2 as described in the legend for Fig. 3. The data are represented as percent survival relative to unstressed cells ($t = 0$ min). The results are the means from at least three independent experiments. Error bars show the standard deviations. For each time, statistically significant differences in survival rates (Student's *t* test; $P < 0.05$) are indicated by different letters (a and b). White bars, BEN2908/pUC13; gray bars, BEN2908/pUC23A; dashed bars, BEN2908 Δ*ibeA*/pUC13 (A) or BEN2908 ΔGimA/pUC13 (B); black bars, BEN2908 Δ*ibeA*/pUC23A (A) or BEN2908 ΔGimA/pUC23A (B). For each time, statistically significant differences in survival rates (Student's *t* test; $P < 0.05$) are indicated by different letters (a, b, and c).

ducible peroxidase OsmC, the periplasmic copper/zinc-dependent superoxide dismutase SodC, the disulfide bond reductase YfcG, the stress response protein YjaA, the paraquat-inducible protein PqiA, and two proteins involved in iron-sulfur cluster assembly and repair, the cysteine desulfurase IscS and the Fe-S transport protein SufA, respectively.

Whereas the levels of transcripts of some genes, like *yjaA*, *pqiA*, *iscS*, and *sufA*, were not significantly affected by the deletion of *ibeA*, we found that the expression of *katE*, *sodC*, *osmC*, and *yfcG* was moderately reduced in the *ibeA* mutant (2.3-, 2.16-, 1.79-, and 1.73-fold, respectively) compared to the wild-type strain (Fig. 5). We next performed these transcription analyses with strains carrying plasmid pUC13 (empty vector) or pUC23A (carrying *ibeA*). The presence of either plasmid did not modify the expression of genes which were downregulated in the *ibeA* mutant (data not shown). Furthermore, in the presence of both plasmids, the differences observed above between *ibeA*$^+$ and *ibeA* mutant strains for *katE*, *sodC*, *osmC*, and *yfcG* were not detected. These results suggest that, during exponential growth, the expression of genes involved in oxidative stress resistance mechanisms is reduced in the Δ*ibeA* mutant. However, the lack of complementation at the transcriptional level indicates that alterations other than these downregulations in gene expression also contribute to the decreased survival of the *ibeA* mutant against oxidative stress.

Alterations of RpoS expression are not responsible for the decreased resistance of strain BEN2908 Δ*ibeA*. RpoS has been described as a potential regulator for the expression of some of the

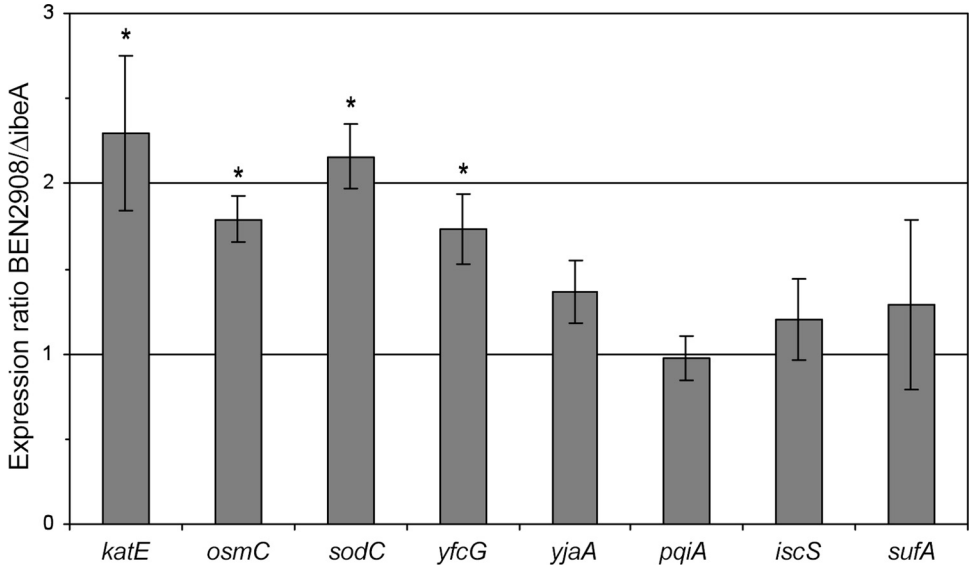

FIG 5 Quantification of the expression of genes involved in the oxidative stress response. Expression of *katE*, *osmC*, *sodC*, *yfcG*, *yjaA*, *pqiA*, *iscS*, and *sufA* in BEN2908 wild-type and Δ*ibeA* strains was measured by quantitative real-time PCR as described in Materials and Methods using the *frr* gene as a housekeeping gene standard. Results are ratios of relative expression in the BEN2908 wild type compared to expression in the Δ*ibeA* mutant. Results are the means from at least three independent experiments. Error bars show the standard deviations. An asterisk indicates that the expression ratio of BEN2908 wild type to Δ*ibeA* was significantly different from 1 ($P < 0.05$ by Student's *t* test).

genes described above. Regulation of gene expression by RpoS mainly involves variations in the cellular level of the RpoS protein (23, 58). We therefore analyzed whether the deletion of *ibeA* had any influence on the amount of RpoS present in the bacteria by Western blotting. Results indicate that the amount of RpoS is not disturbed by the deletion of *ibeA* (Fig. 6A). We used SecG as a control for the amount of protein loaded on the gel. In addition, we investigated whether deletion of *ibeA* had any influence on the motility of strain BEN2908, since RpoS has been shown to regulate the motility of *E. coli* strains by modulating the expression of the master regulator *flhDC* (13, 55). We therefore expected the motility to be modified in case the deletion of *ibeA* had had an effect on the cellular level of RpoS. Clearly, this was not the case. The motilities of strains BEN2908 and BEN2908 Δ*ibeA* were identical (Fig. 6B).

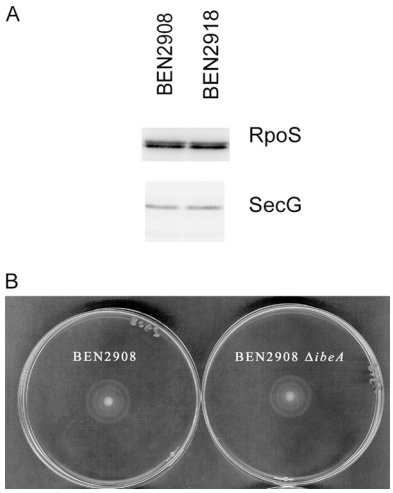

FIG 6 Influence of the *ibeA* deletion on cellular amount of RpoS and motility of strain BEN2908. (A) Bacteria were grown to mid-log phase as described for the H_2O_2 survival assays. The equivalent of 10^8 bacteria per well were loaded on an SDS-PAGE gel, and the amount of RpoS in bacteria was analyzed by Western blotting. (B) Five microliters of a mid-log-phase culture was spotted on an LB agar plate (0.25% agar) and incubated at 37°C for 5 h.

The deletion of *ibeA* does not disturb intracellular redox status. Because of the reduced survival to the oxidative stress response of strain BEN2908 Δ*ibeA*, we tried to determine if this phenotype could be linked to a disruption of the cellular redox potential of bacteria. To this end, we evaluated the proportion of cytoplasmic oxidized disulfide bonds by using an engineered fluorescent protein, rxYFP$^{149}{}_{202}$, as a probe for redox changes (43). In this protein, formation of a disulfide bond between a pair of redox-active cysteines is reversible and results in a >2-fold decrease in the intrinsic fluorescence. Thus, we introduced the plasmid pHOJ124, carrying the gene encoding this reporter protein under the control of an IPTG-inducible promoter, into BEN2908 wild type and Δ*ibeA*. We then determined the rate of oxidized rxYFP$^{149}{}_{202}$ by spectrofluorimetry during mid-exponential growth in M9sup-glucose medium. The proportion of oxidized reporter was similar and very high in the two strains: 82.8% (±5%) and 81.7% (±4%) for BEN2908 wild type and Δ*ibeA*, respectively. Thus, the deletion of *ibeA* does not seem to have any effect on intracellular redox status.

***ibeA*, independently of GimA, is sufficient to increase resistance to H_2O_2 in strain BEN2908.** When *ibeA* is present in an *E. coli* ExPEC strain, it is always located in the structure of the GimA genomic region (24). This suggests that *ibeA* acts in synergy with other genes belonging to GimA. Because GimA contains two other open reading frames (ORFs), *cglE* and *cglD*, also predicted to encode oxidoreductases, it could be that the increased sensitivity of the Δ*ibeA* mutant to H_2O_2 results from an indirect effect of the deletion on the functioning of GimA.

To elucidate this, we constructed a derivative of strain

BEN2908 in which the whole GimA sequence was deleted, and we analyzed its resistance to H_2O_2 in the manner described for Fig. 3A. The ΔGimA mutant presented exactly the same pattern of killing as the Δ*ibeA* mutant, i.e., it showed an important loss of survival compared to the wild-type strain (Fig. 3A). Thus, as was the case for the Δ*ibeA* mutant, the ΔGimA mutant survival rate was 1 and 1.5 logs lower than that of BEN2908 after 40 and 60 min of stress exposure, respectively (Fig. 3A). These results showed that H_2O_2 stress resistance was actually a function performed by GimA.

The ΔGimA mutant was then transformed with either pUC13 or pUC23A, and the survival rates of these strains were monitored during an H_2O_2 challenge (Fig. 4B). As observed for the Δ*ibeA* mutant, the ΔGimA mutant containing pUC13 showed increased sensitivity to H_2O_2 compared to BEN2908 strains transformed with either pUC13 or pUC23A. This difference in survival was significant from 40 min of H_2O_2 stress onwards and reached about 2 logs after 60 min of stress exposure (Fig. 4B). Thus, the expression of *ibeA* alone was able to complement the deleterious impact of GimA deletion on BEN2908 H_2O_2 stress resistance. Therefore, these results showed that *ibeA* alone, without any other GimA component, could improve BEN2908 H_2O_2 stress resistance.

Heterologous expression of *ibeA* in *E. coli* K-12 increases its H_2O_2 stress resistance. We then undertook to determine whether *ibeA* by itself could exert an effect on oxidative stress resistance in a commensal *E. coli* strain. The *E. coli* strain MG1655 was therefore transformed with either pUC13 or pUC23A, and the survival of the two strains was analyzed during an H_2O_2 challenge performed on mid-exponential-growth cultures as previously described. Despite a great variability in survival rates from one experiment to another, *E. coli* MG1655 containing pUC23A, and therefore expressing *ibeA*, always showed greater resistance to H_2O_2 killing than the same strain transformed with the empty vector pUC13. Data from a representative experiment are presented in Fig. 7A. In this experiment, *E. coli* MG1655 containing pUC23A presented 6-fold more CFU after 60 min of stress exposure and thus was significantly more resistant to H_2O_2 than its counterpart carrying the empty vector pUC13 (Fig. 7A). These results show that the *ibeA* gene can confer increased H_2O_2 resistance to a commensal *E. coli* K-12 strain and thus can exert this function in a nonpathogenic background.

We had previously shown that, in strain BEN2908, the expression of type 1 fimbriae was decreased in the BEN2908 Δ*ibeA* mutant (9). It was therefore possible that the increased resistance observed when *ibeA* is expressed in *trans* in *E. coli* K-12 MG1655 is linked to a metabolic alteration due to a modification of type 1 fimbria expression. We investigated this possibility by repeating the H_2O_2 resistance assays with a derivative of strain MG1655 deleted of the *fim* operon. As with strain MG1655, the resistance to H_2O_2 was increased in strain MG1655 Δ*fim* carrying *ibeA* compared to the same strain carrying the empty vector (Fig. 7B). In addition, we showed that expression of the *fimA* promoter was not modified in strain AAEC198A carrying a *fimA*::*lacZ* fusion in the presence of plasmids pUC13 or pUC23A: ß-galactosidase activity (in Miller units) was 201 (±46) and 176 (±35), respectively. We therefore concluded that the influence of the *ibeA* gene on H_2O_2 resistance was independent of the expression of type 1 fimbriae in strain *E. coli* K-12 MG1655.

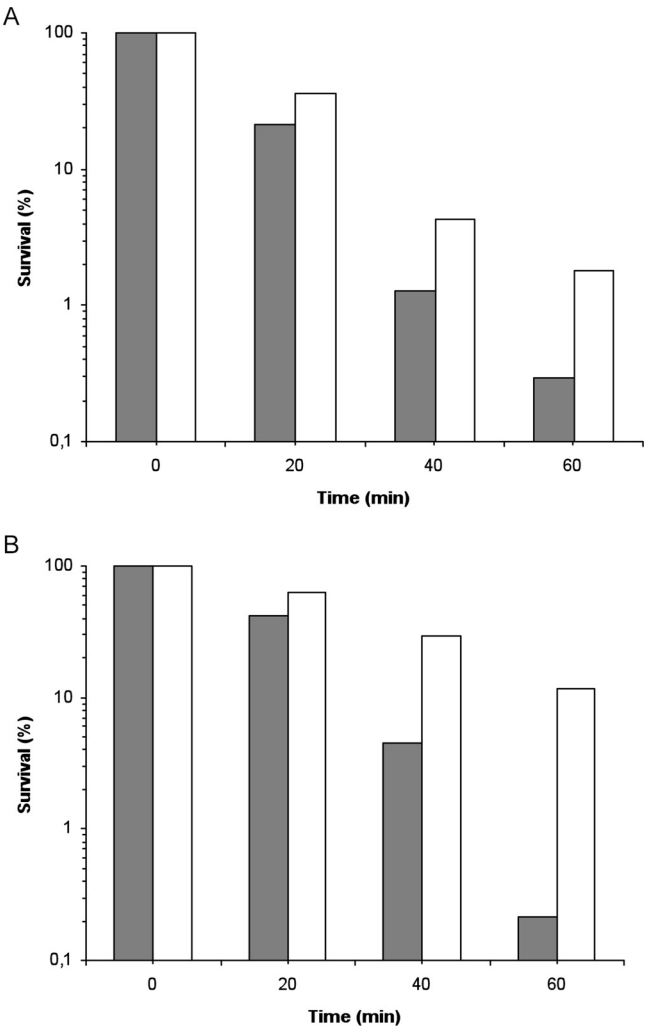

FIG 7 Increased resistance to H_2O_2 killing of strain *E. coli* K-12 MG1655 (A) or MG1655 Δ*fim* (B) containing the *ibeA* gene in *trans*. *E. coli* K-12 strain MG1655 (A) or MG1655 Δ*fim* (B) containing either the pUC13 vector (gray bars) or the pUC23A plasmid expressing the *ibeA* gene (white bars) was treated as described in the legend for Fig. 3. The data are represented as percent survival relative to unstressed cells ($t = 0$ min). For the two strains, the results are representative patterns of survival from at least three independent experiments.

DISCUSSION

Oxidative stress resistance as a new function for *ibeA* in *E. coli*. In this work, we demonstrated that *ibeA* is involved in oxidative stress resistance of *E. coli* BEN2908. Indeed, we showed that a Δ*ibeA* derivative of this strain was significantly more sensitive than its wild-type counterpart during an H_2O_2 challenge, and that the complementation of this mutant by a plasmid expressing *ibeA* restored wild-type resistance to H_2O_2 killing.

In ExPEC strains, *ibeA* belongs to a 20.3-kb genomic island called GimA (25). This island contains 14 genes in addition to *ibeA*, some of which are predicted to encode proteins involved in carbon source metabolism and stress resistance. *ibeR*, which belongs to the same operon as *ibeA*, was studied in the meningitic strain *E. coli* E44. In this strain, an *ibeR* mutant is more sensitive to various stresses, including H_2O_2 killing (6). Thus, it could be possible that IbeR, which is annotated as a transcriptional regulator, is

responsible for *ibeA* induction and, thus, for increased oxidative stress resistance in ExPEC strains.

A recent study established that, when present in an ExPEC strain, GimA is always complete, except in some cases in which a 342-bp remnant is found (24). Thus, it can be supposed that *ibeA* interacts with other GimA components in an organized system. Among the other components of GimA, there are two other genes predicted to encode oxidoreductases: *cglD*, coding for a putative glycerol dehydrogenase, and *cglE*, encoding a protein sharing strong similarities with IbeA. It was thus possible that the deletion of *ibeA* led to an imbalance in oxidoreductase expression within GimA, which could have been responsible for the oxidative stress sensitivity phenotype. Such a possibility was ruled out by the observation that, first, a BEN2908 derivative deleted for the whole GimA genomic region presented the same increased sensitivity to H_2O_2 as the Δ*ibeA* mutant and that, second, the expression of *ibeA* alone in a strain lacking the entire GimA island was able to restore survival after H_2O_2 stress to a level similar to that of the wild-type strain. As a consequence, the lower resistance to H_2O_2 killing of the Δ*ibeA* mutant seemed to reflect a real function brought by the *ibeA* gene rather than an experimental artifact.

To reinforce these observations, we added *ibeA* in *trans* to the *E. coli* strains MG1655 and MG1655 Δ*fim* that both lack GimA. The resulting strains presented significantly increased H_2O_2 stress resistance compared to their counterpart transformed with the empty vector. Taken as a whole, our data show that *ibeA* alone, without any other GimA components, is actually sufficient to confer increased H_2O_2 stress resistance to *E. coli*.

The oxidative stress response is altered in the Δ*ibeA* mutant during exponential growth. Although these results bring new light to the contribution of IbeA to *E. coli* physiology, the question of its exact function still remains. The analysis of conserved domains in IbeA revealed extended similarities, encompassing an FAD binding domain, to members of the FixC family that have been found in diverse species and are thought to contribute to electron transfer reactions (16, 17). We thus propose that IbeA protein is involved in some oxidoreductive mechanisms. This suggestion is corroborated by the cytoplasmic localization of the protein that we also demonstrated in this study by different subcellular localization experiments. This feature is in accordance with all of the predictions that we obtained in a previous work using several dedicated software programs (9). Nevertheless, it is in contradiction to previous studies assigning to IbeA a direct adhesive function (59, 60). To verify our hypothesis, it is now essential that the precise reaction mediated by IbeA be determined. For this purpose, we intend to overexpress and purify IbeA to characterize its *in vitro* activity in future experiments.

As *ibeA* is implicated in H_2O_2 stress resistance in BEN2908, we investigated, in the Δ*ibeA* mutant, the possibility of expression alterations of several genes involved in the oxidative stress response of *E. coli*. Among the genes tested, we identified four that were moderately downregulated in the mutant compared to the wild-type strain during exponential growth in minimal medium: *katE*, *sodC*, *osmC*, and *yfcG*. During aerobic growth, *E. coli* produces sufficient H_2O_2 to create toxic levels of DNA damage via the Fenton reaction (44). KatE, the monofunctional catalase hydroperoxidase II, is implicated, in association with KatG and AhpCF, in *E. coli* H_2O_2 scavenging and detoxification (44). The osmotically inducible peroxidase OsmC is able to detoxify organic hydroperoxides and, to a much lesser extent, inorganic hydrogen peroxide (36). Thus, an *E. coli osmC* mutant is sensitive to exposure to hydrogen peroxide and tert-butylhydroperoxide (8). The periplasmic copper/zinc-dependent superoxide dismutase SodC is responsible for superoxide anion degradation. A mutation in the gene *sodC* also leads to an increased sensitivity to H_2O_2 in *E. coli* (22). Finally, the disulfide bond reductase YfcG has a glutathione (GSH)-conjugating activity as well as a GSH-dependent peroxidase activity, and a mutation in the *yfcG* gene decreases the resistance to H_2O_2 in *E. coli* (30). However, expression data obtained with strains containing the *ibeA*$^+$ plasmid pUC23A or the empty vector pUC13 failed to demonstrate a complementation of these expression data. This suggests that alterations of expression of these four genes could only partly explain the sensitivity to H_2O_2 killing of the Δ*ibeA* mutant. It is more likely that other properties of the Δ*ibeA* mutant are responsible for alterations in oxidative defense mechanisms during the exponential growth which sensitize bacteria to a subsequent H_2O_2 stress exposure.

The four genes *katE*, *sodC*, *osmC*, and *yfcG* belong to the RpoS regulon in different *E. coli* strains (4, 22, 52, 57). More specifically, *katE* and *yfcG* were shown to be under the control of *rpoS* during *E. coli* exponential growth in glucose-containing M9 medium (57), i.e., under the experimental conditions used in this study, suggesting that RpoS-dependent stress response is at least partly affected in the Δ*ibeA* mutant. Interestingly, Chi et al. proposed that IbeR was a functional equivalent of RpoS in *E. coli* E44 that presents a loss-of-function mutation in the *rpoS* gene (6). However, our results demonstrated that the cellular level of RpoS was not affected by the *ibeA* mutation, and that motility, a phenotype linked to RpoS, was not disturbed in the *ibeA* mutant. It is therefore unlikely that the increased sensitivity to H_2O_2 of the *ibeA* mutant is linked to a modification in RpoS levels.

The downregulation of genes involved in oxidative stress resistance in the Δ*ibeA* mutant also suggests that the cellular redox state of this strain could be affected. Nevertheless, we were unable to show any difference between BEN2908 wild-type and Δ*ibeA* mutant strains by performing a spectrofluorimetric analysis of the two strains transformed with pHOJ124 (43), a vector expressing a yellow fluorescent protein whose fluorescence intensity depends on the redox state of the cell.

How could the increased H_2O_2 sensitivity of the Δ*ibeA* mutant affect BEN2908 virulence? In this work, we showed that *ibeA* is implicated in H_2O_2 stress resistance of *E. coli* BEN2908. However, the exact contribution of this phenotype to the virulence of strain BEN2908 still has to be explored. During the infection process of colibacillosis, which is mainly a disease initiated in the respiratory tract, we can identify at least two steps during which the bacteria are exposed to an oxidative stress: the response of the host's immune system and the survival phase in the respiratory tract environment.

The first step corresponds to an oxidative burst produced by chicken macrophages and heterophils after bacteria phagocytosis (11, 42). This consists of a cocktail of several reactive oxygen and nitrogen species, including superoxide anions, hydrogen peroxide, and nitric oxide, which has a biocidal action on bacteria (11, 42). Thus, bacteria's ability to resist this defense mechanism will influence their survival rate inside the host and thus have an effect on their pathogenic potential. To determine the effect of *ibeA* and GimA deletions on *E. coli* BEN2908 intramacrophage survival, we performed survival challenges in the macrophage cell lines RAW264.7 (murine) and HD11 (avian). We have never been able

to show any intramacrophage survival differences between BEN2908 and BEN2908 Δ*ibeA*, suggesting that *ibeA* and, more globally, GimA are not involved in this infection step (data not shown).

The second hypothesis that we considered, i.e., the involvement of *ibeA* in bacterial survival in the respiratory tract environment, cannot be easily addressed by any experimental procedure. Nevertheless, an experimental reproduction of colibacillosis in chicken previously performed in our laboratory suggested that *ibeA* was involved early in the infection of chickens by strain BEN2908 (20). The lung environment presents a high oxygen tension, which could lead to a higher rate of production of reactive oxygen species, including hydrogen peroxide. Thus, the *ibeA* gene of *E. coli* BEN2908 could be responsible for an increased resistance of the bacteria to these harsh living conditions, allowing them to survive well enough to cause the disease. Such a situation has already been described for the virulence gene *phgA* of *Streptococcus pneumoniae*, which is responsible for pneumonia. In this case, *phgA* is also involved in resistance to H_2O_2 killing, but the authors were unable to show any involvement of this gene in the survival of the phagocytic respiratory burst, suggesting rather that *phgA* takes part in oxidative stress resistance in the lung environment (5). Finally, it was shown that *E. coli* survival inside nonphagocytic epithelial cells also requires protection against oxidative damage, especially a high level of SodC (1). Thus, it is possible that the Δ*ibeA* mutant, whose *sodC* gene is downregulated, is less able to survive within respiratory epithelial cells.

In conclusion, results of this work shed new light on the still ill-characterized exact function of IbeA. These results, together with sequence data and previous reports in the literature, let us hypothesize that IbeA is involved in an oxidoreduction reaction that could modify the behavior of the bacteria regarding expression of virulence genes or resistance to oxidative stress. The nature of the substrate on which IbeA would act remains to be identified and deserves further study.

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