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MOLECULAR PATHOGENESIS

Salmonella enterica Serovars Dublin and Enteritidis Comparative Proteomics Reveals Differential Expression of Proteins Involved in Stress Resistance, Virulence, and Anaerobic Metabolism

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ABSTRACT The Enteritidis and Dublin serovars of *Salmonella enterica* are phylogenetically closely related yet differ significantly in host range and virulence. *S.* Enteritidis is a broad-host-range serovar that commonly causes self-limited gastroenteritis in humans, whereas *S.* Dublin is a cattle-adapted serovar that can infect humans, often resulting in invasive extraintestinal disease. The mechanism underlying the higher invasiveness of *S.* Dublin remains undetermined. In this work, we quantitatively compared the proteomes of clinical isolates of each serovar grown under gut-mimicking conditions. Compared to *S.* Enteritidis, the *S.* Dublin proteome was enriched in proteins linked to response to several stress conditions, such as those encountered during host infection, as well as to virulence. The *S.* Enteritidis proteome contained several proteins related to central anaerobic metabolism pathways that were undetected in *S.* Dublin. In contrast to what has been observed in other extraintestinal serovars, most of the coding genes for these pathways are not degraded in *S.* Dublin. Thus, we provide evidence that *S.* Dublin metabolic functions may be much more affected than previously reported based on genomic studies. Single and double null mutants in stress response proteins Dps, YciF, and YgaU demonstrate their relevance to *S.* Dublin invasiveness in a murine model of invasive salmonellosis. All in all, this work provides a basis for understanding interserovar differences in invasiveness and niche adaptation, underscoring the relevance of using proteomic approaches to complement genomic studies.

KEYWORDS comparative proteomics, *Salmonella* Dublin, *Salmonella* Enteritidis, stress resistance, virulence, anaerobic metabolism

The genus *Salmonella* is of clinical importance because it is one of the main causative agents of food-transmitted diseases globally. It is estimated that nontyphoidal *Salmonella* (NTS) causes a burden of 154 million cases per year worldwide (1). On the other hand, it is estimated that 3.4 million cases of invasive NTS (iNTS) diseases occur annually, with a case fatality rate of 20%, yielding about 680,000 deaths per year globally (2). More than 2,600 serovars have been described for *Salmonella enterica*; however, only around 50 are regularly isolated from humans (3, 4). *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) is one of the main NTS serovars involved in human infections globally, usually causing a limited gastroenteritis in healthy individuals (5, 6). However, other less prevalent NTS serovars, such as *Salmonella enterica* serovar Dublin (*S.* Dublin), frequently cause invasive (extraintestinal) infections with higher morbidity and mortality (4). Thus, *S.* Dublin shows a substantially higher invasive index than *S.* Enteritidis globally (33% for *S.* Dublin, 1.8% for *S.* Enteritidis) (6). In addition, these two serovars differ in their

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host ranges. While *S.* Enteritidis can infect a broad range of hosts, including humans, poultry, and cattle, *S.* Dublin is adapted to cattle but still able to infect humans (7). In spite of their notable phenotypic differences, serovars Dublin and Enteritidis are very closely related (7). Comparative genomic analyses revealed few genes of difference between the two serovars (8–10). In this sense, Mohammed and Cormican found that two different type VI secretion systems encoded on *Salmonella* pathogenicity islands 6 and 19 (SPI-6 and SPI-19), a Gifsy-2-like prophage and a virulence plasmid, are present in *S.* Dublin but absent in *S.* Enteritidis, and they suggested that all contribute to *S.* Dublin's high capacity of causing invasive disease in humans (11). Fenske et al. also identified a type VI secretion protein, VgrG, and a type I fimbrial subunit, FimI, as virulence factors present in *S.* Dublin but absent in *S.* Enteritidis (10). However, crucial differences may exist at the gene expression level, resulting in differential abundance of proteins, data that are not revealed using comparative genomic analyses.

In addition, several lines of evidence indicate that a major driving force of evolution toward host adaptation and an extraintestinal lifestyle is genome degradation through pseudogenization and gene loss. We and others have previously demonstrated that *S.* Dublin genomes contain more instances of degraded genes than *S.* Enteritidis genomes (7–9). Furthermore, extensive genome decay was observed in other host-restricted/adapted serovars that cause extraintestinal infections, such as *S.* Typhi, *S.* Paratyphi A, *S.* Paratyphi C, *S.* Gallinarum, and *S.* Choleraesuis (12–15). Moreover, *S.* Enteritidis and *S.* Typhimurium invasive lineages that emerged in sub-Saharan Africa show more genomic degradation than their globally isolated counterparts (16, 17).

Salmonella can interact with the host intestinal epithelium and invade it using a specialized type three secretion system (SPI-1 T3SS), triggering an acute inflammatory response. It is generally accepted that the evolution of the disease is partially dependent on the expression of bacterial factors during the intestinal phase of the infection (6, 18). Thus, studying the *Salmonella* proteome under conditions mimicking those encountered in the gut, though not a perfect replica of the *in vivo* environment, provides valuable insights into which proteins are produced that could be relevant for *Salmonella* invasive ability. Despite a study analyzing the proteome of *S.* Typhimurium grown under these conditions (19), no proteomic comparison between differently invasive *Salmonella* serovars grown under an infection-relevant condition has been reported.

In this work, we performed a large-scale comparative proteomic profiling of one clinical isolate from each serovar (Dublin and Enteritidis), grown under gut-mimicking conditions (GMC), i.e., high osmolarity, low oxygen tension, and the presence of biliary salts and short-chain fatty acids. Among more than 2,000 proteins identified in total, we detected nearly 560 differentially represented in the proteomes of both serovars. These included a high proportion of proteins involved in environmental stress resistance and virulence overrepresented in serovar Dublin. Conversely, we found numerous proteins involved in chemotaxis and central anaerobic metabolism present in *S.* Enteritidis but undetected in the *S.* Dublin proteome. Furthermore, we provide evidence that natural isolates of *S.* Dublin are more resistant to oxidative and acid stress conditions than those of *S.* Enteritidis *in vitro* and demonstrate that Dps and the *Salmonella* uncharacterized proteins YgaU and YciF contribute to serovar Dublin *in vivo* invasiveness.

RESULTS AND DISCUSSION

Comparative proteomic analysis: serovars Dublin and Enteritidis express different sets of protein functions under GMC. Using liquid chromatography-tandem mass spectrometry (LC-MS/MS), we performed a comparative proteomic analysis between one selected isolate of each serovar, SDu3 (here termed SDU) and SEn8/02 (here termed SEN). Proteins exclusively detected in each serovar were pinpointed using the statistical module included in Protein for Proteomics software ($P < 0.05$), while a protein was considered overexpressed in one serovar when the enrichment was 1.5-fold or higher with a P value <0.05. Here, we will collectively refer to overrepresented

proteins as the sum of exclusively detected and overexpressed proteins in one serovar compared to the other.

The total numbers of proteins identified in each serovar, considering at least its presence in two replicates, were 1,782 and 1,888 for SDU and SEN, respectively (see Table S1 in the supplemental material); from these, 151 or 201 proteins were exclusively detected in SDU or SEN, respectively (Fig. 1A and Table S1). Among the proteins detected in both serovars, 117 were significantly overexpressed in SDU compared to SEN (rendering a total of 268 proteins overrepresented in SDU), while 93 proteins were significantly overexpressed in SEN compared to SDU (rendering a total of 294 proteins overrepresented in SEN) (Fig. 1A and Table S1). Of note, only 17 of 151 proteins exclusively detected in SDU do not have homologous genes in SEN (Table S1), underscoring the relevance of analyzing not only genomes but also proteomes in comparative studies.

The gene ontology (GO) functional enrichment analysis of differentially represented proteins between both serovars is shown in Fig. 1B and Table S2. Among the GO terms enriched in the SDU overrepresented proteins with a *P* value lower than 0.05, several are linked to the response to different types of stress. We refined the search manually and found 29 proteins reported or annotated as related to stress response that were overrepresented in SDU versus SEN (Table 1, Fig. 1C). Twenty-four out of these 29 proteins are controlled by the alternative sigma factor RpoS that regulates a global adaptive response allowing survival in starvation and under various environmental stresses (Table 1) (20). Consistent with this, RpoS levels were found to be 5.1-fold higher in SDU than SEN. As this factor is induced upon entrance to stationary phase or in the presence of diverse stresses (21), we verified that both strains (SDu3 and SEn8/02) were at the same phase of the curve when collecting bacteria despite being at different values of optical density at 600 nm (OD_{600}) (Fig. S1). In addition, we quantified the number of CFU/ml at the same time point when the cells were collected for proteomic analysis and found that for both strains the CFU numbers were equivalent ($4.73 \times 10^8 \pm 0.79 \times 10^8$ and $6.05 \times 10^8 \pm 1.22 \times 10^8$ CFU/ml for SDu3 and SEn8/02, respectively). Moreover, we measured mRNA levels for *rpoS* at different time points along the growth curve and found that the induction kinetics is the same for both strains (Fig. S2). Thus, differences in expression of stress-related genes due to differences in growth phase could be discarded.

In addition, several proteins associated with *Salmonella* pathogenicity islands (SPIs) or the virulence plasmid revealed statistically higher abundance in SDU than SEN (Table 2, Fig. 1D). Among these, several components of the SPI-1 T3SS, regulators (including HilA, the activator of SPI-1 expression) and secreted effectors were found. *Salmonella* employs this T3SS to invade enterocytes through a process of bacterium-mediated endocytosis, disrupting the intestinal mucosal barrier.

Collectively, all of these features could account for the higher invasive capacity observed for *S.* Dublin than for *S.* Enteritidis. Interestingly, Huang and colleagues reported that the expression of many virulence proteins in SPI-1 T3SS was significantly higher in *S.* Choleraesuis (a highly adapted and extraintestinal serovar) than in *S.* Typhimurium (a ubiquitous and gastrointestinal serovar) grown in RPMI cell culture medium (22). Moreover, 14 of the 29 stress-related proteins found in the present work as overrepresented in SDU compared to SEN were previously reported by Huang et al. as overexpressed in *S.* Choleraesuis compared to *S.* Typhimurium (Table 1). These results suggest a common way to achieve high invasiveness in both extraintestinal serovars.

We also found several proteins involved in flagellar biosynthesis, encoded by class 1 and 2 flagellar genes, overrepresented in SDU (Table S3) (23). This was surprising, since we previously demonstrated that SDu3 strain lacks flagella due to a permanent inhibition of FliA, the specific sigma factor for class 3 flagellar genes, rendering expression of these genes silenced (24). Indeed, several proteins encoded by class 3 genes, such as FliC and CheABMRVWYZ, were found overrepresented in the SEN proteome

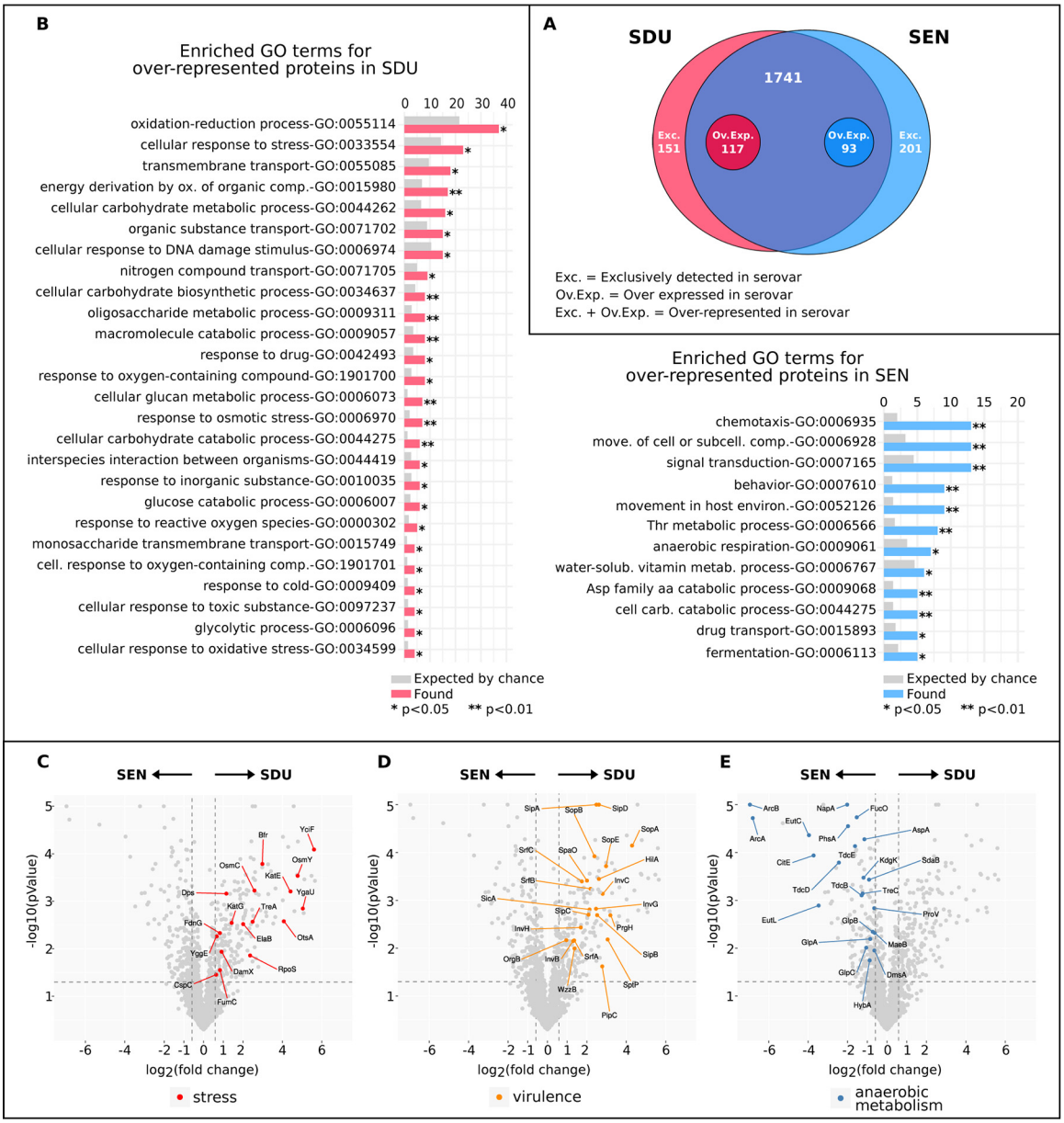

FIG 1 (A) Venn diagram showing the proteins detected by LC-MS/MS in isolates SDu3 (left) and SEn 8/02 (right). Proteins exclusively detected in SDU or SEN (151 and 201 for SDU and SEN, respectively) were identified using the PatternLab for Proteomics Approximately Area Proportional Venn Diagram module ($P < 0.05$). Among the 1,741 shared proteins, 117 are overexpressed in SDU versus SEN and 93 are overexpressed in SEN versus SDU (smaller inserted circles). Table S1 shows the list of total proteins identified in at least two replicates of each serovar (1,782 and 1,888 for SDU and SEN, respectively). (B) Gene ontology functional enrichment analysis of proteins overrepresented in SDU compared to SEN (pink bars) or vice versa (light-blue bars). Terms in the category "Biological Process" with enrichment *P* values <0.05 compared to the background group (all detected proteins in at least two replicates of the serovar) are shown. Gray bars indicate the protein counts expected if they were present in the analyzed group by chance, annotated with each GO term. Pink or light-blue bars indicate the counts found in the analyzed group (overrepresented proteins in *S.* Dublin or *S.* Enteritidis, respectively), annotated with each GO term. *P* values are shown as asterisks at the right of each bar. (C to E) Volcano plots of proteins detected in both serovars. The fold change values were calculated with PatternLab's TFold module, considering at least four biological replicates of both serovars. The logarithmic ratios of average fold changes are reported on the *x* axis. The *y* axis shows negative logarithmic *P* values obtained from the *t* test. The threshold values for *P* value (0.05) and fold change (1.5) are indicated by dashed lines. (C and D) Proteins involved in stress resistance (C) and virulence (D) overexpressed in SDU versus SEN are indicated with red and orange dots, respectively. (E) Proteins involved in anaerobic metabolism overexpressed in SEN versus SDU are indicated with blue dots. Note that proteins exclusively detected in each serovar are not depicted in the Volcano plots.

TABLE 1 Proteins related to stress response overrepresented in SDU versus SEN[d]

Locus name in P125109[a]	Gene name[b]	Description	Fold change[c]	*P* value
SEN1303	*katN**	Manganese-containing catalase	++	<0.05
SEN1075	***otsB****	Trehalose-6-phosphate phosphatase	++	<0.05
SEN1607	***sodC****	Superoxide dismutase (Cu-Zn) precursor	++	<0.05
SEN1674	*sufC**	Iron-sulfur cluster assembly ATPase protein SufC	++	<0.05
SEN1673	*sufD**	Iron-sulfur cluster assembly protein SufD	++	<0.05
SEN1672	*sufS**	Cysteine desulfurase, SufS subfamily	++	<0.05
SEN3426	***treF****	Alpha-alpha-trehalase, cytoplasmic trehalase	++	<0.05
SEN1304	*yciE**	Ferritin-like domain-containing protein	++	<0.05
SEN1536	***ydeI****	YdeI family stress tolerance OB fold protein	++	<0.05
SEN2331	*yfcG**	Probable glutathione *S*-transferase	++	<0.05
SEN2920	*yggG**	Putative metalloprotease YggG	++	<0.05
SEN3104	***yhbO****	General stress protein 18	++	<0.05
SEN1305	***yciF****	Stress response diiron-containing protein, in *Salmonella* induced in bile	48.573	8.143E−05
SEN2639	***ygaU****	Conserved hypothetical protein, peptidoglycan binding LysM domain	32.708	0.0014
SEN4323	***osmY****	Osmotically inducible protein OsmY	27.317	2.910E−04
SEN1725	***katE****	Catalase/hydroperoxidase II, HPII	21.263	6.198E−04
SEN1076	***otsA****	Alpha, alpha-trehalose-phosphate synthase (UDP-forming)	16.795	0.0026
SEN3271	*bfr**	Bacterioferritin	7.892	1.637E−04
SEN1492	***osmC****	Osmotically inducible protein C	5.969	5.927E−04
SEN1241	*treA**	Alpha, alpha-trehalase, periplasmic trehalase	5.596	0.0027
SEN2763	*rpoS**	RNA polymerase sigma factor	5.129	0.0138
SEN2293	***elaB****	Conserved hypothetical protein ElaB	4.016	0.0030
SEN3900	***katG***	Catalase/hydroperoxidase HPI	2.677	0.0028
SEN0776	***dps****	DNA-binding protein Dps/iron-binding ferritin-like antioxidant protein/ferroxidase	2.223	6.894E−04
SEN3311	*damX*	DamX, an inner membrane protein involved in bile resistance	1.876	0.0115
SEN1579	*fumC*	Fumarate hydratase class II	1.778	0.0280
SEN_RS19920	*fdnG*	Formate dehydrogenase O alpha subunit, selenocysteine containing	1.769	0.0047
SEN2908	*yggE**	Oxidative stress defense protein, DUF541	1.585	0.0055
SEN1200	*cspC*	Cold shock protein CspC	1.552	0.0351

[a]The genome annotation of *S.* Enteritidis strain P125109 was used as the reference (GenBank accession no. AM933172.1, NCBI RefSeq accession no. NC_011294.1).
[b]Boldface indicates genes coding for proteins reported by Huang et al. (22) as overexpressed in *S.* Choleraesuis compared to *S.* Typhimurium. An asterisk indicates genes positively controlled (directly or indirectly) by the alternative sigma factor RpoS (genes downregulated in the Δ*rpoS* mutant compared to the wild-type strain, $P < 0.05$, according to Lévi-Meyrueis [20]).
[c]++, protein was exclusively detected in SDU.
[d]All proteins included in this table were identified through at least two peptides in at least two replicates in the LC-MS/MS data analysis.

compared to SDU (S3 table). Thus, in the absence of class 3 gene expression, class 1 and 2 flagellar genes may be overexpressed in SDU in an attempt to compensate for the impairment of late flagellar gene expression.

Among the proteins overrepresented in SEN compared to SDU, the GO enrichment analysis revealed many involved in chemotaxis, movement, amino acids, and carbohydrate metabolism and anaerobic respiration, among other biological processes (Fig. 1B and Table S2). Proteins involved in chemotaxis and motility overrepresented in SEN include FliC, Aer, Tsr, McpC, and CheABMRVWYZ, as mentioned above (Table S3). It has been demonstrated that chemotaxis and motility are important for intestinal colonization but are not required for systemic infection (25–28). Therefore, our results are in accordance with the notion that these phenotypes are expressed in *S.* Enteritidis because they are required for its intestinal environment-adapted lifestyle, whereas they may be dispensable for *S.* Dublin.

On the other hand, we found a striking number (55) of proteins involved in central anaerobic metabolism overrepresented in SEN (Table 3, Fig. 1E). These include those required for vitamin B_{12} biosynthesis, galactose transport, and utilization of arginine, citrate, ethanolamine, 1,2-propanediol, idonate, and fucose, among others. The majority were undetected in SDU but present in SEN. Interestingly, it has been reported that in the context of an inflamed gut, *S.* Typhimurium can metabolize ethanolamine and 1,2-propanediol through anaerobic respiration, using alternative electron acceptors, such as nitrate or tetrathionate, which confers an advantage over the competing microbiota (26, 29–31). We also found proteins involved in the reduction of nitrate or

TABLE 2 Proteins involved in virulence overrepresented in SDU versus SEN[c]

Virulence locus and gene name	Locus name in P125109[a]	Description	Fold change[b]	*P* value
SPI-1				
hilA	*SEN2718*	Type III secretion transcriptional activator HilA	6.1	3.60E−04
invA	*SEN2737*	Type III secretion inner membrane channel protein	++	<0.05
invB	*SEN2736*	Type III secretion system protein BsaR; surface presentation of antigens protein SpaK (invasion protein InvB)	2.47	7.10E−03
invC	*SEN2735*	Type III secretion cytoplasmic ATP synthase (FlII/YscN family ATPase)	6.97	7.30E−04
invE	*SEN2738*	Type III secretion outer membrane contact-sensing protein invasion protein InvE	++	<0.05
invG	*SEN2739*	Type III secretion outer membrane pore forming protein	5.52	1.50E−03
invH	*SEN2741*	Invasion protein InvH precursor	3.23	3.70E−03
orgB	*SEN2711*	OrgB protein, associated with InvC ATPase of type III secretion system	1.94	6.81E−03
prgH	*SEN2716*	Type III secretion protein EprH	9.14	2.05E−03
sicA	*SEN2727*	Type III secretion chaperone protein	4.39	1.55E−03
sicP	*SEN2721*	Secretion chaperone (associated with virulence)	++	<0.05
sipA	*SEN2723*	Type III secretion injected virulence protein	5.59	1.00E−05
sipB	*SEN2726*	Cell invasion protein SipB	5.74	2.04E−03
sipC	*SEN2725*	Cell invasion protein SipC (effector protein)	4.24	2.00E−03
sipD	*SEN2724*	Type III secretion host injection protein	6.1	1.00E−05
sitA	*SEN2703*	Manganese ABC transporter, periplasmic binding protein SitA	++	<0.05
sitB	*SEN2704*	Manganese ABC transporter, ATP-binding protein SitB	++	<0.05
spaO	*SEN2732*	Type III secretion inner membrane protein	4.02	3.90E−04
sprB	*SEN2708*	SPI1-associated transcriptional regulator SprB	++	
sptP	*SEN2720*	SPI-1 type III secretion system effector GTPase-activating protein SptP	8.3	6.58E−03
SPI-5				
sigE or pipC	*SEN0954*	Type III secretion system chaperone SigE	6.86	2.40E−02
sopB	*SEN0955*	Inositol phosphate phosphatase SopB	5.22	1.20E−04
Prophage SE12				
sopE	*SEN1155*	SPI-1 type III secretion system guanine nucleotide exchange factor SopE	7.86	1.90E−04
Virulence plasmid				
spvA	*pSENV_002*	Outer membrane protein, virulence protein SpvA	++	<0.05
spvB	*pSENV_003*	SPI-2 type III secretion system effector NAD(+)-protein-arginine ADP-ribosyl transferase SpvB	++	<0.05
spvC	*pSENV_004*	Type III secretion system effector phospho threonine lyase	++	<0.05
spvD	*pSENV_005*	SpvD type III secretion effector	++	<0.05
Other virulence factors				
asmA	*SEN2116*	Outer membrane assembly protein AsmA	++	<0.05
slrP	*SEN_RS03860*	SPI-1 type III secretion system effector E3 ubiquitin transferase SlrP	++	<0.05
sopA	*SEN2065*	SPI-1 type III secretion system effector HECT-type E3 ubiquitin transferase SopA	19.58	7.00E−05
sopD	SEN2784	SPI-1 type III secretion system effector SopD	++	<0.05
sopE2	*SEN1182*	G-nucleotide exchange factor SopE2	++	<0.05
srfA	*SEN1462*	Putative virulence effector protein (SsrAB-activated protein)	2.56	6.89E−03
srfB	*SEN1461*	Putative virulence factor SrfB	4.55	5.70E−04
srfC	*SEN1460*	Putative virulence effector protein SrfC	3.35	4.00E−04
wzzB	*SEN2078*	Regulator of length of O-antigen component of lipopolysaccharide chains	2.59	1.01E−02

[a]The genome annotation of *S.* Enteritidis strain P125109 was used as the reference (GenBank accession no. AM933172.1, NCBI RefSeq accession no. NC_011294.1).
[b]++, protein was exclusively detected in SDU.
[c]All proteins included in this table were identified through at least two peptides in at least two replicates in the LC-MS/MS data analysis.

tetrathionate, such as NapA or TtrA, respectively, overrepresented in SEN compared to SDU. Moreover, both ethanolamine and 1,2-propanediol utilization pathways require the anaerobically synthesized vitamin B_{12} as a cofactor (30), and CbiK and CobD, two enzymes involved in *de novo* vitamin B_{12} biosynthesis, were also found among the proteins exclusively detected in SEN. Thus, under the growth conditions assessed here, SEN possesses a plethora of enzymes relevant for proliferating in the anaerobic gut environment that are undetected or underexpressed in SDU.

Remarkably, Nuccio and Baumler reported that a network of 469 coding DNA sequences (CDSs) involved in central anaerobic metabolism underwent a high degree of degradation in extraintestinal compared to gastrointestinal *Salmonella* serovars (15).

TABLE 3 Proteins involved in central anaerobic metabolism overrepresented in SEN versus SDU[c]

Locus name in P125109[a]	Gene name	Description	Fold change[b]	*P* value
SEN2530	*asrC*	Anaerobic sulfite reductase subunit C	++	<0.05
SEN4218	*arcA*	Arginine deiminase, ADI	112.27	1.91E−05
SEN4216	*arcB*	Ornithine carbamoyltransferase, catabolic OCT	124.12	1.00E−05
SEN4217	*arcC*	Carbamate kinase, CK	++	<0.05
SEN4214	*arcD*	Arginine/ornithine antiporter ArcD	++	<0.05
SEN4221	*argI*	Ornithine carbamoyltransferase	++	<0.05
SEN4213	*argR*	Arginine pathway regulatory protein ArgR, repressor of *arg* regulon	++	<0.05
SEN4096	*aspA*	Aspartate ammonia-lyase	2.22	5.29E−05
SEN2023	*cbiK*	Sirohydrochlorin cobaltochelatase CbiK, vitamin B_{12} biosynthesis	++	<0.05
SEN0053	*citB*	Transcriptional regulatory protein CitB	++	<0.05
SEN0592	*citD*	Citrate lyase gamma chain, acyl carrier protein CitD	++	<0.05
SEN0591	*citE*	Citrate lyase beta chain CitE	13.23	1.15E−04
SEN0590	*citF*	Citrate lyase alpha chain CitF	++	<0.05
SEN0587	*citT*	Citrate succinate antiporter CitT	++	<0.05
SEN0613	*cobD*	L-Threonine 3-O-phosphate decarboxylase CobD	++	<0.05
SEN0869	*dmsA*	Anaerobic dimethyl sulfoxide reductase chain A	1.56	1.12E−02
SEN1551	*dmsA1-STM1499*	Anaerobic selenate reductase, molybdenum cofactor-containing periplasmic protein	++	<0.05
SEN1555	*dmsD*	Anaerobic dimethyl sulfoxide reductase chaperone DmsD	++	<0.05
SEN2439	*eutA*	Ethanolamine utilization protein EutA	++	<0.05
SEN2438	*eutB*	Ethanolamine ammonia-lyase heavy chain EutB	++	<0.05
SEN2437	*eutC*	Ethanolamine ammonia-lyase light chain EutC	15.6	4.35E−05
SEN2446	*eutD*	Phosphate acetyl transferase EutD, ethanolamine utilization-specific	++	<0.05
SEN2443	*eutE*	Acetaldehyde dehydrogenase, ethanolamine utilization cluster EutE	++	<0.05
SEN2441	*eutG*	Ethanolamine utilization protein EutG	++	<0.05
SEN2436	*eutL*	Ethanolamine utilization polyhedral-body-like protein EutL	11.1	1.28E−03
SEN2445	*eutM*	Ethanolamine utilization polyhedral-body-like protein EutM	++	<0.05
SEN2444	*eutN*	Ethanolamine utilization polyhedral-body-like protein EutN	++	<0.05
SEN2448	*eutQ*	Ethanolamine utilization protein EutQ	++	<0.05
SEN2447	*eutT*	ATP:cob(I)alamin adenosyltransferase, ethanolamine utilization, EutT	++	<0.05
SEN4110	*frdD*	Fumarate reductase subunit D	++	<0.05
SEN3835	*fucO*	Lactaldehyde dehydrogenase involved in fucose or rhamnose utilization, lactaldehyde reductase	2.90	1.84E−05
SEN2266	*glpA*	Anaerobic glycerol-3-phosphate dehydrogenase subunit A	1.80	6.42E−03
SEN2267	*glpB*	Anaerobic glycerol-3-phosphate dehydrogenase subunit B	1.65	4.54E−03
SEN2268	*glpC*	Anaerobic glycerol-3-phosphate dehydrogenase subunit C	2.08	9.79E−03
SEN1251	*hyaA*	Uptake (NiFe) hydrogenase, small subunit HyaA	1.51	4.74E−04
SEN2992	*hybA*	Hydrogenase-2 operon protein hybA precursor	1.84	1.80E−02
SEN4237	*idnD*	L-Idonate 5-dehydrogenase IdnD	++	<0.05
SEN4236	*idnO*	5-Keto-D-gluconate 5-reductase IdnO	++	<0.05
SEN3435	*kdgK*	2-Dehydro-3-deoxygluconate kinase	2.29	3.36E−04
SEN2451	*maeB*	NADP-dependent malic enzyme	1.54	4.79E−03
SEN2183	*mglB*	Galactose/methyl galactoside ABC transport system, D-galactose-binding periplasmic protein MglB	++	<0.05
SEN2242	*napA*	Periplasmic nitrate reductase precursor, catalytic subunit NapA	4.06	1.00E−05
SEN2037	*pduB*	Propanediol utilization polyhedral body protein PduB	++	<0.05
SEN2038	*pduC*	Propanediol dehydratase large subunit PduC	++	<0.05
SEN2039	*pduD*	Propanediol dehydratase medium subunit PduD	++	<0.05
SEN2063	*phsA*	Thiosulfate reductase precursor	3.94	2.82E−05
SEN2654	*proV*	L-Proline glycine betaine ABC transport system permease protein ProV	1.57	1.46E−03
SEN2815	*sdaB*	L-Serine dehydratase, beta subunit/L-serine dehydratase, alpha subunit	1.9	3.70E−04
SEN3086	*tdcA*	Threonine catabolic operon transcriptional activator TdcA	++	<0.05
SEN3085	*tdcB*	Threonine dehydratase, catabolic, L-serine dehydratase, PLP-dependent	3.08	7.37E−05
SEN3083	*tdcD*	Propionate kinase	5.43	1.63E−04
SEN3082	*tdcE*	2-Ketobutyrate formate-lyase/pyruvate formate-lyase	2.44	7.86E−04
SEN4204	*treC*	Trehalose-6-phosphate hydrolase	2.36	7.21E−04
SEN1662	*ttrA*	Tetrathionate reductase subunit A	++	<0.05
SEN4152	*ulaD*	3-Keto-L-gulonate-6-phosphate decarboxylase UlaD (L-ascorbate utilization protein D)	++	<0.05

[a]The genome annotation of *S.* Enteritidis strain P125109 was used as the reference (GenBank accession no. AM933172.1, NCBI refseq accession no. NC_011294.1).
[b]++, protein was exclusively detected in SEN.
[c]All proteins included in this table were identified through at least two peptides in at least two replicates in the LC-MS/MS data analysis.

They hypothesized that genes that promote growth in the distal gut are dispensable to extraintestinal serovars because they are adapted to thrive in host systemic tissues, not the gut. Moreover, Langridge et al. reported that host-adapted serovars, such as Gallinarum, Pullorum, and Dublin, show metabolic deficiencies compared to Enteritidis, and these deficiencies are correlated to their pseudogene content (7). Specifically, the pathways they demonstrated to be affected in *S.* Dublin were those related to D-glucarate and L-arabinose degradation as well as galactoside transport. Genomic analyses conducted by Nuccio and Baumler and Langridge et al. demonstrated an intermediate amount of degraded genes in *S.* Dublin compared to the host-restricted serovars Gallinarum, Pullorum, and Typhi and the ubiquitous serovars Enteritidis and Typhimurium (7, 15). In this work, however, based on proteomic results, we provide evidence that *S.* Dublin has much more affected metabolic functions than previously reported based on genomic studies (7, 15). We wondered whether this could be due to increased degradation of CDSs involved in these functions in the SDu3 isolate compared to *S.* Dublin isolates reported previously. Thus, we analyzed the genomic sequence of SDu3 and found that except for *mglB* (encoding a galactose/methyl galactoside ABC transport system), none of the genes coding for the differentially represented proteins shown in Table 3 are disrupted. We also analyzed the sequence of genes encoding proteins that directly regulate the expression of *cit*, *eut*, *pdu*, *idn*, and *arc* operons and found that, with the exception of *dpiB* (coding for the operon *cit* regulator), the corresponding genes are not inactivated (Fig. S3). Moreover, sequence alignment revealed minor differences in amino acid sequences for these proteins between both serovars (Fig. S3), suggesting that a regulatory mechanism is responsible for the impaired expression in *S.* Dublin isolates. It is tempting to speculate that this represents an intermediate stage in the process of gene loss in extraintestinal *Salmonella* serovars in which the genes required for thriving in the intestine are silenced as a step before their inactivation.

Interestingly, the upregulation of proteins involved in anaerobic fumarate respiration and 1,2-propanediol and arginine utilization was previously reported in the proteome of *S.* Typhimurium grown under gut-mimicking conditions compared to standard laboratory conditions (19). In our study, this seems to be the case for SEN but not for SDU, supporting the hypothesis that the gastrointestinal serovars are better adapted to grow in the gut anaerobic environment than the extraintestinal ones.

mRNA levels correlate with proteomic results. To test if proteomic differences were related to transcriptional regulation, we selected 10 proteins overrepresented in each serovar to measure their mRNA levels in SDu3 and SEn8/02 and in 3 additional isolates of each serovar (Table 4), grown under the same conditions as those used for the proteomic analysis. Among proteins overrepresented in SDU, we selected 6 genes putatively involved in response to osmotic, oxidative, or acid stresses (*dps*, *katN*, *osmY*, *yciE*, *yciF*, and *ygaU*) and *rpoS*, one coding for a secreted virulence factor (*sopA*), one coding for a lipopolysaccharide modification enzyme (*lpxR*), and a gene coding for a protein involved in fructose phosphorylation and transport through the cell membrane (*fruF*). Among proteins overrepresented in SEN, we selected 8 genes encoding proteins involved in central anaerobic metabolism (*eutB*, *eutM*, *pduB*, *pduC*, *ttrA*, *adi*, also named *arcA*, *oct*, also named *arcB*, and *ck*, also named *arcC*), one encoding an enzyme involved in ribonucleic acid degradation (*cpdB*), and one encoding a peptidyl-prolyl *cis-trans* isomerase (*fklB*).

As shown in Fig. 2A and B, 17 of 20 genes tested exhibited mRNA levels significantly different between serovars that are consistent with the proteomic results, and all strains belonging to the same serovar behaved similarly.

Remarkably, mRNA levels for the 8 proteins involved in central anaerobic metabolism were significantly more abundant in SEN (Fig. 2B), suggesting that the differences found at the proteomic levels are due to inhibition of transcription and/or increased mRNA degradation in SDU.

In the case of the 6 proteins involved in stress response overrepresented in SDU, all of them also showed mRNA levels significantly higher in SDU (Fig. 2A) despite *rpoS*

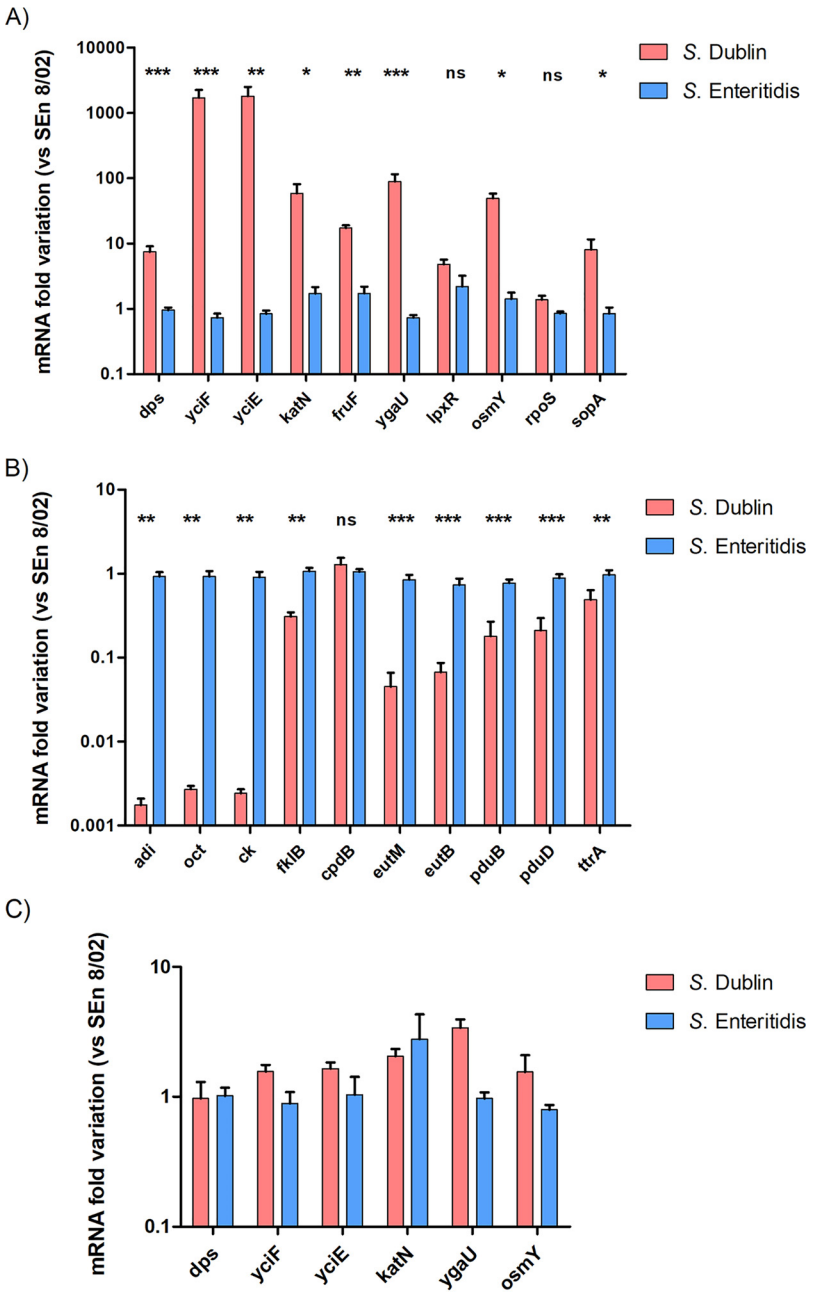

FIG 2 mRNA level quantification of genes coding for selected proteins differentially represented between *S.* Dublin and *S.* Enteritidis. Four isolates of each serovar grown under GMC (A and B) or in LB with aeration (C) were analyzed. (A) Genes coding for proteins overrepresented in SDu3 compared to SEn8/02. (B) Genes coding for proteins overrepresented in SEn8/02 compared to SDu3. (C) Genes coding for proteins involved in stress response overrepresented in SDu3 versus SEn8/02; isolates were grown in LB with aeration. For real-time analysis, we used the $2^{-\Delta\Delta CT}$ method for relative mRNA quantitation, using *icdA* as the normalizing gene. The isolate SEn8/02 was arbitrarily selected as the calibrator condition; thus, the RNA levels depicted in the graphs for both groups (*S.* Dublin or *S.* Enteritidis) are related to SEn8/02 levels. Results are the means ± standard errors of the means (SEM) from two or three independent experiments, with four strains in each group. Note that the *y* axis is in logarithmic scale. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not statistically significant (Mann-Whitney test). In panel C, no significant differences were found between serovars for each gene.

mRNA levels were not statistically different between the serovars. We also measured mRNA levels for these 6 genes under a stress condition other than GMC (presence of 0.5 mM hydrogen peroxide) and found significantly higher levels in SDU for 5 of 6 genes (Fig. S4). However, when the isolates were grown under standard laboratory

conditions (LB broth with aeration at 200 rpm), the differences in mRNA levels were not significant, indicating that differential expression is specifically displayed when bacteria are stress exposed (Fig. 2C). These results suggest that serovar Dublin responds more efficiently than *S.* Enteritidis to stressful environmental conditions, inducing the expression of stress-related genes to a higher extent.

***dps* and *yciF* null mutants are impaired for *in vitro* stress resistance.** To investigate if any of the proteins overrepresented in SDU compared to SEN contribute to its invasiveness, we selected 3 postulated as being involved in stress responses (Dps, YciF, and YgaU) to inactivate the corresponding genes in the chromosome of one strain of each serovar. Dps (**D**NA-binding **p**rotein from **s**tarved cells) is a conserved ferritin-like protein involved in DNA protection against oxidative, thermal and acid stress that has been reported to be required for *S.* Typhimurium survival in macrophages and for full virulence in mice (32). Interestingly, Dps has been localized in the cytosol but also in the outer membrane, suggestive of moonlighting activities (33). YciF is encoded in an RpoS-regulated operon (*yciGFE-katN*), which, in *S.* Typhimurium, is induced in the presence of bile (20, 34). Recently, it was reported to be involved in regulating the outer membrane porins and cell permeability (35). YgaU is a protein of unknown function in *Salmonella*, annotated as a peptidoglycan-binding protein containing a LysM domain and also positively regulated by RpoS (20). Its orthologue in *Escherichia coli* (also named Kbp) is a K^+ binding protein postulated as a sensor of cytoplasmic K^+ concentration that influences peptidoglycan cross-linking under envelope stress conditions (36, 37). In addition, the orthologous Dps, YciF, and YgaU proteins from *E. coli* are induced by osmotic stress (38). The fold changes in abundance of these proteins in the SDU proteome related to SEN were the following: (Dps, 2.22; YciF, 48.57; YgaU, 32.71) (Table 1). In all three cases, the mRNA levels were also significantly higher in SDU when grown under GMC (Fig. 2A).

Single null mutants were constructed in the Dps, YciF, and YgaU coding genes in isolates SDu3 and SEn8/02. The mutants were tested in *in vitro* assays of resistance against oxidative, acid, and osmotic stresses.

As shown in Fig. 3A, *dps* inactivation led to a significant decrease in hydrogen peroxide (H_2O_2) resistance compared to that of the parental strain in both serovars, consistent with previous results observed for *S.* Typhimurium (32). Inactivation of *yciF* or *ygaU* did not significantly affect survival under this condition in any serovar. Interestingly, the SDU wild-type (WT) isolate exhibited significantly higher tolerance to oxidative stress conditions than the wild-type SEN isolate (the percentage of survival after 2 h in 5 mM H_2O_2 was 25.24 and 0.94% for SDu3 and SEn8/02, respectively).

Regarding resistance to acidic pH, in serovar Dublin inactivation of *dps*, *yciF*, or *ygaU* showed no significant difference compared to the wild-type isolate at any time point (Fig. 3B). For serovar Enteritidis, the *yciF* mutant was significantly more sensitive to acidic pH than the wild-type strain after 1 h of acid challenge, whereas the *dps* and *ygaU* mutants showed no phenotype (Fig. 3B).

We also tested resistance to 2 h of incubation with 0.5 mM H_2O_2 and 3 and 6 h of incubation at pH 3.1 of 4 natural isolates of each serovar and demonstrated that collectively, *S.* Dublin is significantly more resistant than *S.* Enteritidis in both assays (Fig. S5).

Concerning resistance to high osmolarity (0.8 M NaCl), difference was found between neither the mutants and the parental strains nor the wild-type strains of both serovars (Fig. 3C).

In summary, we demonstrate that *in vitro*, Dps has a relevant role in protection against oxidative stress in SDU and SEN and YciF has a role in resistance to acid stress in SEN, whereas YgaU inactivation has no effect on resistance to the tested stresses.

Moreover, our results strongly suggest that natural isolates of serovar Dublin are more resistant to environmental stress conditions (such as those found during infection of host tissues) than *S.* Enteritidis isolates, which could contribute to its higher

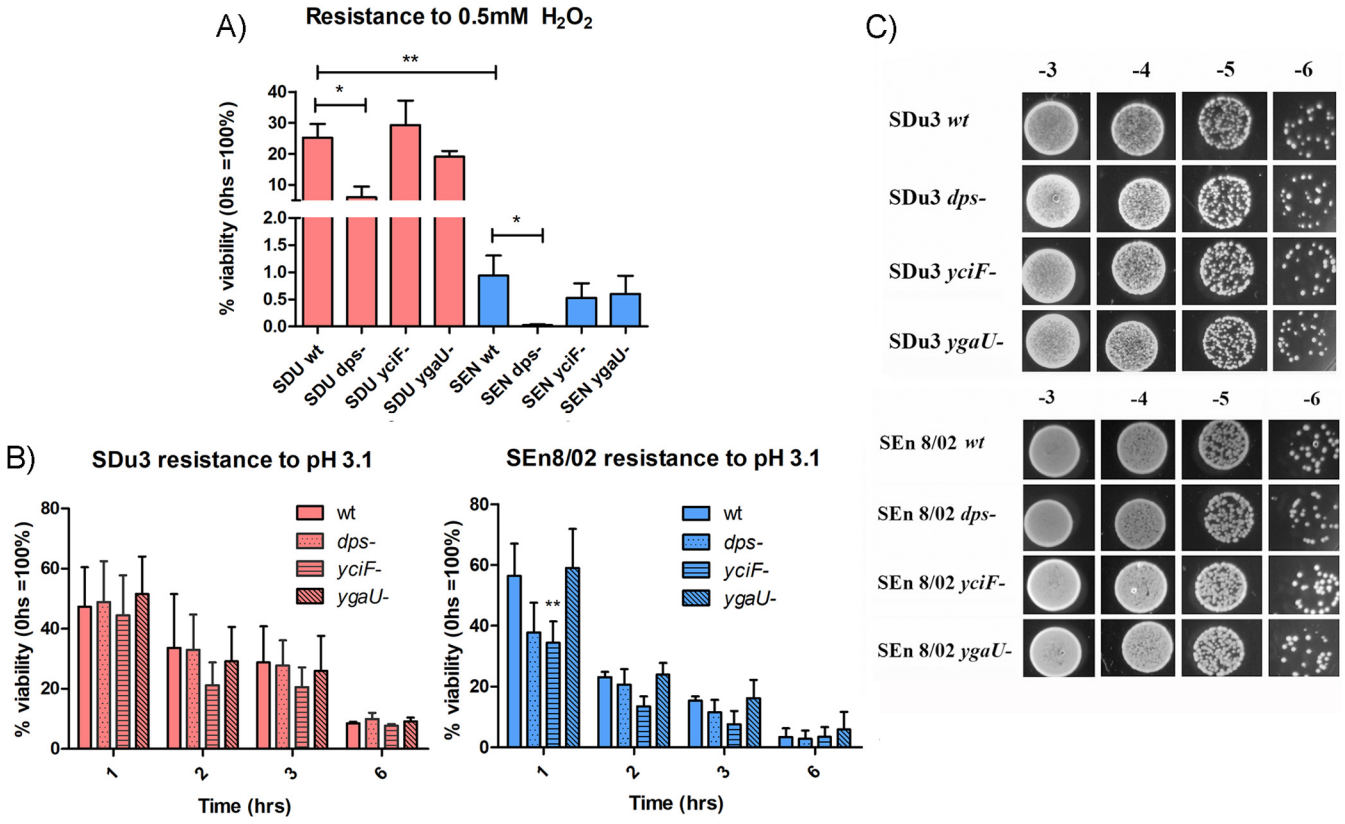

FIG 3 *In vitro* stress resistance assays of SDu3 and SEn8/02 and the corresponding *dps*, *yciF*, and *ygaU* null mutant derivatives. (A) Oxidative stress tolerance assay. Surviving bacteria are expressed as a percentage of the initial inoculum after 2 h of exposure to 0.5 mM hydrogen peroxide at 37°C and represent the average percent survival compared to 0 h exposure from three independent experiments. Error bars represent the standard errors of the means. (B) Acid stress tolerance assay. Results are expressed as percent survival after 1, 2, 3, and 6 h of exposure to pH 3.1 at 37°C and represent the average percent survival from three independent experiments. Error bars represent the standard errors of the means. *, $P < 0.05$; **, $P < 0.01$ (Mann-Whitney test). (C) Osmotic stress tolerance assay. Serial dilutions of overnight cultures of SDu3 (top) and SEn 8/02 (bottom) and the *dps*, *yciF*, and *ygaU* null mutant derivatives were spotted onto LB agar plates supplemented with 0.8 M NaCl and incubated for 24 h at 37°C.

invasiveness. In this sense, the requirement of an acid resistance response to survive the harsh acidic conditions of the stomach or inside the *Salmonella*-containing vacuole once the bacteria become intracellular has been reported. Further, for a successful systemic infection, *Salmonella* must withstand phagocyte oxidative burst and the antimicrobial response of the host innate immune response (39, 40).

Role of *dps*, *yciF*, and *ygaU* in *Salmonella* invasiveness in mice. It has been demonstrated that *S.* Dublin is significantly more virulent in terms of systemic colonization than *S.* Enteritidis in mice (41), recapitulating what is observed in humans. We also verified that the SDu3 isolate reaches significantly higher bacterial counts in spleens of mice than SEn8/02 in the mouse model of invasive salmonellosis (Fig. S6).

To investigate if the inactivated genes have a role in *Salmonella in vivo* invasiveness, the *dps*, *yciF*, and *ygaU* null mutants were evaluated in mice in competition assays with the corresponding wild-type strains. A mixture containing equal amounts of mutant and parental strains was intragastrically inoculated into BALB/c mice, and 4 days post-infection (p.i.), bacterial counts in spleens were determined. Mutants were distinguished from the corresponding wild-type strain because of their antibiotic (kanamycin or chloramphenicol) resistance phenotype.

In the SDu3 isolate, neither mutation affected virulence (Fig. 4A). In SEn8/02, single inactivation of *dps* significantly attenuated virulence, while inactivation of *yciF* or *ygaU* showed no phenotype related to the wild-type strain (Fig. 4A).

It was previously reported that *dps* has a role in *S.* Typhimurium virulence, because a null mutant showed less capacity to reach/colonize internal organs in

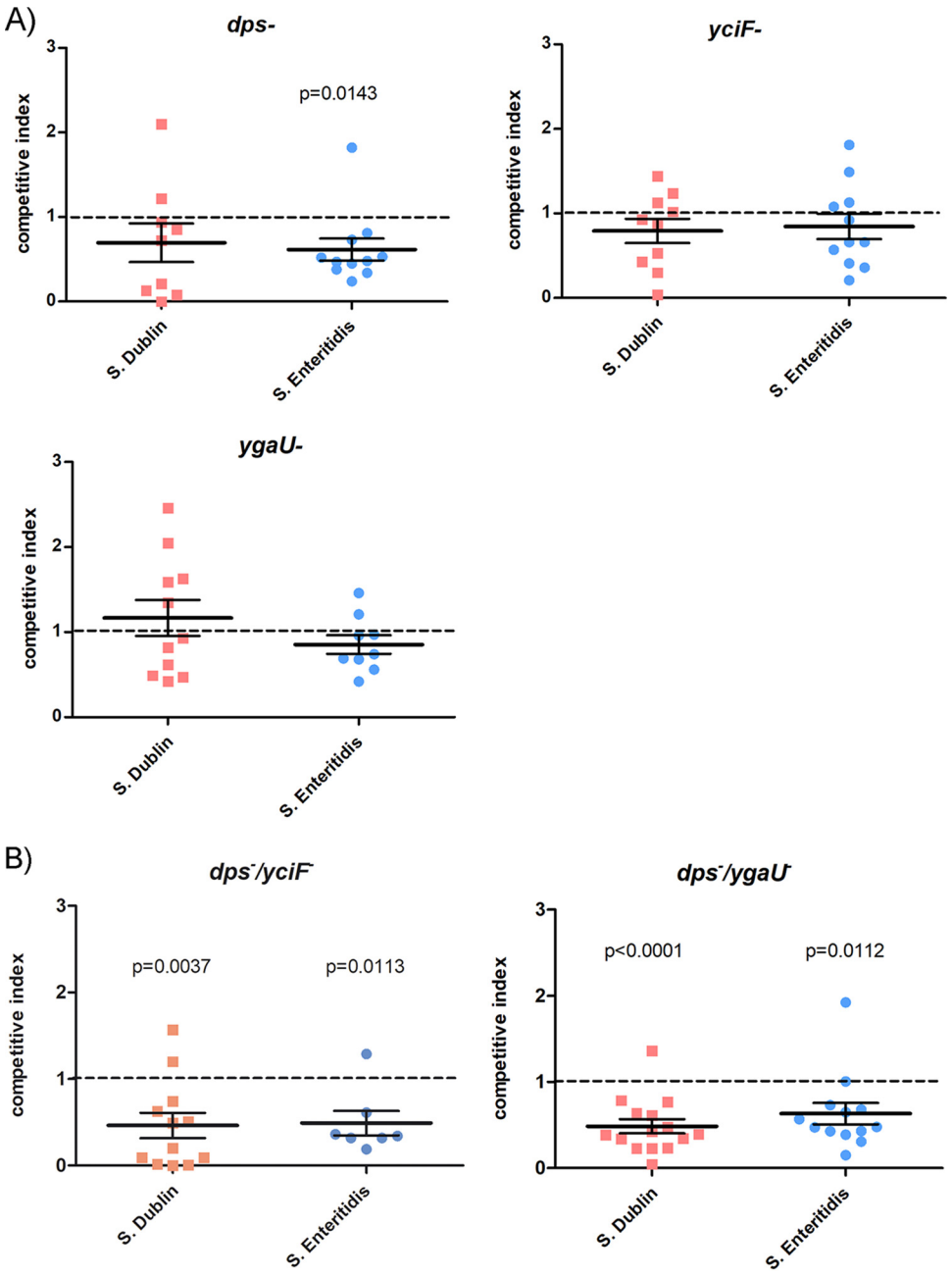

FIG 4 Competitive index of SDU and SEN single and double null mutants in the murine model of invasive salmonellosis. (A) CI of individual null mutants. Mixtures (1:1) of the wild type (SEn8/02 and SDu3) and the corresponding mutants (either *dps*::*cat*, *yciF*::*kan*, or *ygaU*::*kan*) were administered intragastrically to BALB/c mice (2×10^6 to 5×10^6 CFU/mouse). Mice were sacrificed 4 days p.i., spleens removed, and dilutions plated onto LB agar for CFU counting. At least 100 colonies per organ were replica plated onto LB agar containing chloramphenicol or kanamycin and LB agar without antibiotics. The CI was determined by dividing the ratio of mutant to wild-type bacteria in the spleen by the ratio of mutant to wild-type bacteria in the inoculum. Each point is the CI from one mouse, and the dashed line indicates a competitive index of 1. Statistical significance for each group being different than 1 was calculated using a one-sample, two-tailed *t* test (GraphPad Prism). Results from two independent experiments are shown, including means ± SEM. (B) CI of double mutants (either *dps*::*cat-yciF*::*kan* or *dps*::*cat-ygaU*::*kan*). The assay and the CI determination were as described for panel A and were from two or three independent experiments. When the group mean is statistically different from 1, the *P* value is indicated. No *P* value indicates that the difference is not significant.

TABLE 4 *S*. Dublin and *S*. Enteritidis natural isolates, reference strains, and mutant strains used in this work

Strain or isolate	Description[a]	Source and/or reference
S. Dublin		
SDu1	Human blood isolate (1995)	28
SDu3	Human blood isolate (2006)	28
SDu5	Human feces isolate (2000)	28
SDu6	Human feces isolate (2005)	28
SDu3 *dps*::*cat*	SDu3 derivative containing *dps*::*cat* (Cm^r)	This work
SDu3 *yciF*::*kan*	SDu3 derivative containing *yciF*::*kan* (Kan^r)	This work
SDu3 *ygaU*::*kan*	SDu3 derivative containing *ygaU*::*kan* (Kan^r)	This work
SDu3 *dps*::*cat-yciF*::*kan*	SDu3 derivative containing *dps*::*cat* (Cm^r) and *yciF*::*kan* (Kan^r)	This work
SDu3 *dps*::*cat-ygaU*::*kan*	SDu3 derivative containing *dps*::*cat* (Cm^r) and *ygaU*::*kan* (Kan^r)	This work
S. Enteritidis		
SEn 31/88	Human feces isolate (1988)	46
SEn 8/89	Human blood isolate (1989)	46
SEn 251/01	Chicken egg isolate (2001)	46
SEn 8/02	Human feces isolate (2002)	46
P125109 (PT4)	Reference strain of *S*. Enteritidis	14
SEn 8/02 *dps*::*cat*	SEn 8/02 derivative containing *dps*::*cat* (Cm^r)	This work
SEn 8/02 *yciF*::*kan*	SEn 8/02 derivative containing *yciF*::*kan* (Kan^r)	This work
SEn 8/02 *ygaU*::*kan*	SEn 8/02 derivative containing *ygaU*::*kan* (Kan^r)	This work
SEn 8/02 *dps*::*cat-yciF*::*kan*	SEn 8/02 derivative containing *dps*::*cat* (Cm^r) and *yciF*::*kan* (Kan^r)	This work
SEn 8/02 *dps*::*cat-ygaU*::*kan*	SDu3 derivative containing *dps*::*cat* (Cm^r) and *ygaU*::*kan* (Kan^r)	This work

[a]The year of isolation is indicated in parentheses.

mice than the wild-type strain (32). In addition, Wright et al. demonstrated that several genes involved in protection against oxidative damage are induced in *S*. Typhimurium inside phagocytes in response to the oxidative burst. Individual mutants, however, with the exception of *dps*, were not affected in mice (42). Our data confirmed a relevant role of *dps* for *S*. Enteritidis virulence, whereas for the extraintestinal serovar Dublin, a dispensable role was shown despite its significantly increased sensitivity to oxidative stress conditions observed *in vitro*. This result suggests that *in vivo*, other proteins would compensate for the Dps lack in oxidative stress resistance, leading to no phenotype of the *S*. Dublin *dps* single mutant.

On the other hand, the absence of phenotype of the *ygaU* mutant, both *in vitro* and *in vivo*, is surprising. Rosenkrantz et al. analyzed in *S*. Typhimurium the transcriptional response to 8 different environmental stress conditions and found that *ygaU* had a significantly different expression under all conditions evaluated compared to the control condition (43). This gene was overexpressed under acid, oxidative, osmotic, and heat shock stress conditions as well as in the stationary phase of growth, and it was repressed in exponential phase and anaerobiosis. Notably, they failed to construct a null mutant in this gene, so they could not assess the importance of *ygaU* in stress adaptation or virulence. However, in an evaluation of an *S*. Typhimurium random transposon mutant library in BALB/c mice, *ygaU* was found to be dispensable for systemic virulence (44).

Due to possible redundant functions of the inactivated genes, we constructed the double null mutants *dps-yciF* and *dps-ygaU*. As shown in Fig. 4B, all double mutants were significantly attenuated in virulence in both serovars. This result suggests that, in serovar Dublin, *dps* has a redundant role in virulence with *yciF* and *ygaU*, because single mutants showed no phenotype, but when two genes (*dps-yciF* or *dps-ygaU*) were inactivated simultaneously, the resulting strains were significantly affected in the capacity to reach/colonize internal organs compared to the wild-type strain. On the contrary, in *S*. Enteritidis, inactivation of *yciF* or *ygaU* does not seem to decrease virulence beyond that of the *dps* strain (Fig. 4A and B).

Collectively, these results suggest that SDU harbors more redundancy of functions related to environmental stress resistance than SEN. In addition, we conclude that the

uncharacterized *Salmonella* proteins YgaU and YciF, and the previously characterized stress protein Dps, have a contributing role in *S*. Dublin virulence in mice.

However, deletion of *dps-yciF* or *dps-ygaU* in SDu3 does not equate its virulence to that of SEn8/02 (note that the difference in spleen bacterial counts between wild-type isolates SDu3 and SEn8/02 is more than 1 order of magnitude [Fig. S6], while the CIs of the SDu3 double mutants are around 0.5 [Fig. 4B]). These results indicate that the difference in invasiveness observed *in vivo* for these serovars is a result of multiple factors in addition to those studied here.

Conclusions. Here, we conducted a comprehensive comparative proteomic analysis between clinical isolates of *Salmonella enterica* serovars Dublin and Enteritidis grown under infection-relevant (gut-mimicking) conditions. The majority of detected proteins constitute a core proteome of 1,741 proteins, while 151 and 201 were exclusively detected in SDU and SEN, respectively. Our results reveal a significant amount of proteins involved in stress resistance, virulence, anaerobic metabolism, and motility differentially represented between both serovars, data that were not previously inferred from comparative genomic analysis (7–9, 15). This outlines the relevance of analyzing the proteomes as a complement to the genomic studies when comparing closely related *Salmonella* serovars in search of the molecular basis of differential phenotypes. Moreover, by analyzing four isolates of each serovar, we found that mRNA levels were consistent with the proteomic results for 17 of the 20 genes analyzed, with little intraserovar variation. Differences found in the mRNA levels for stress response genes under GMC disappeared when bacteria were grown under standard laboratory conditions, highlighting the relevance of using a growth condition that simulates intrahost environment for the proteomic studies.

Based on the results of the comparative proteomics, stress resistance tests, and mutational analysis presented here, we hypothesize that strains of serovar Dublin are better prepared to deal with the harsh environmental conditions found in host tissues. This feature, together with the increased expression of proteins involved in the invasion of host cells, may enhance *S*. Dublin's capacity to cause invasive infections compared to *S*. Enteritidis. In a previous work in which the proteomes of *S*. Choleraesuis and *S*. Typhimurium were compared, the authors found several stress-related and virulence proteins overrepresented in the former (22). Thus, it is tempting to speculate that a similar mechanism operates in *S*. Choleraesuis and *S*. Dublin for achieving higher invasiveness than the gastrointestinal serovars Typhimurium and Enteritidis.

Finally, in this work we demonstrate that, under gut-mimicking conditions, several proteins related to anaerobic metabolism are silenced in an extraintestinal and host-adapted compared to a gastrointestinal and ubiquitous *Salmonella* serovar despite the presence of the corresponding active coding genes in both. We hypothesize that this particular proteomic pattern of the SDU isolate is indicative of decreased metabolism in the anaerobic environment of the gut. Our data reinforce the notion that invasive serovars are defective for growth in the intestinal environment compared to gastrointestinal serovars (15) and may point to a mechanism of silencing genes no longer needed for growth in the systemic environment. This may represent an intermediate stage in the process of evolution toward host adaptation and an extraintestinal lifestyle.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Uruguayan *Salmonella* isolates were obtained from the National *Salmonella* Centre (NSC) and the Bacteriology Unit of the Ministry of Public Health (MPH) collections (Table 4).

Luria-Bertani (LB) broth and LB agar (Miller, Sigma) were used for routine aerobic cultures at 37°C in an orbital shaking incubator (200 rpm) with kanamycin (50 μg/ml), chloramphenicol (25 μg/ml), or ampicillin (100 μg/ml) added when required. Bacterial stocks were maintained frozen at –80°C in LB containing 16.7% glycerol.

To mimic the environmental conditions of the gastrointestinal tract *in vitro*, *Salmonella* isolates were grown overnight, nonagitated, at 37°C in 3 ml of LB broth. The cultures then were diluted 50-fold in fresh LB supplemented with 25 mM sodium acetate, 3 μM sodium deoxycholate, 0.2 M sodium chloride

(added to the NaCl contained in the LB broth; final concentration of 0.37 M), pH 7.0, and grown at 37°C, nonagitated, to the mid-exponential phase of growth. The tubes were half filled and the caps were tightly closed to maintain low aerobic conditions. This condition is referred to as the gut-mimicking condition (GMC) (19, 45).

Protein sample preparation. Isolates SDu3 and SEn8/02 were selected as representatives of Dublin and Enteritidis serovars, respectively. SDu3 was isolated in 2006 from an invasive human infection and SEn8/02 in 2002 from a case of gastroenteritis (Table 4). Both isolates have been extensively characterized (24, 28, 46, 47), and their genomic sequences were obtained.

To collect bacteria from both serovars at the same phase of the growth curve, we performed growth curves under GMC to determine the mid-exponential phase for both strains. *S*. Dublin grew at a lower rate and reached lower OD_{600} values at stationary phase than *S*. Enteritidis (see Fig. S1 in the supplemental material). Therefore, OD_{600} of ~0.35 and ~0.5 were determined for mid-exponential growth phase under GMC for *S*. Dublin and *S*. Enteritidis, respectively (around 4.5 h of growth under GMC). At these OD_{600} values, bacteria were collected for CFU number quantification (by plating phosphate-buffered saline [PBS] serial dilutions of the cultures in LB agar) and protein extract preparation for proteomic analysis. Bacterial cultures were centrifuged at 2,700 × *g*, 10°C, resuspended in 25 mM Tris-HCl, pH 8, and centrifuged again, and the pellet was dissolved in lysis buffer (7 M urea, 2 mM thiourea, 10 mM Tris-HCl, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, pH 8) (two-dimensional [2D] electrophoresis, principles and methods; GE Healthcare). The samples then were frozen at −80°C and quickly thawed/frozen three times, sonicated, and centrifuged again to remove unbroken cells. The protein concentration in the supernatants was determined using a 2D Quant kit (GE Healthcare). A 2D clean-up kit from GE Healthcare was used to remove contaminating substances.

Proteomic analysis. Three independent biological replicates of each serovar were analyzed.

Aliquots containing 20 μg of each protein extract were run on 1-cm-long SDS gels (12.5% acrylamide). In-gel Cys alkylation was performed by incubation with 10 mM dithiothreitol for 1 h at 56°C, followed by incubation with 55 mM iodoacetamide at room temperature in the absence of light for 1 h. In-gel digestion was performed overnight at 37°C by incubation with trypsin (sequencing grade; Promega). Peptide extraction was performed with 0.1% trifluoroacetic acid (TFA) in 60% acetonitrile for 1 h at 30°C with shaking. Samples were vacuum dried, resuspended in 0.1% TFA, sonicated 5 min three times, and desalted using C_{18} OMIX tips (Agilent). Peptides were eluted with acetonitrile–0.1% formic acid, vacuum dried, and resuspended in 0.1% formic acid.

LC-MS/MS analysis. Tryptic peptides were separated using a nano-high-performance liquid chromatograph (HPLC) (UltiMate 3000; Thermo Scientific) coupled with a Q-Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific). Two technical replicates of each sample were analyzed. Peptide mixtures were injected into an Acclaim PepMap 100 C_{18} HPLC column (75 μm by 2 cm; Thermo Scientific) and separated on a reverse-phase C_{18} analytical column (75-μm diameter, 500 mm long, 2-μm particle size, 100-Å pore size; Easy-Spray column ES803; Thermo Scientific). The multistep gradient at 0.2-μl/min flow with solvent A (0.1% formic acid) and solvent B (0.1% formic acid in 80% acetonitrile) was 1% B from 0 to 15 min, increasing to 50% B from 15 to 195 min, increasing to 99% B from 195 to 210 min, and then holding at 99% B until 220 min. The mass spectrometer was operated in a data-dependent mode, switching from full MS to MS/MS acquisition. Full MS spectra were acquired across a mass range of 200 to 2,000, at a resolution of 70,000, and tandem mass spectra of the 12 most intense peaks were recorded using a dynamic exclusion list (resolution of 17,500 and stepped collision energy of 25, 30, and 35).

LC-MS/MS data processing. LC-MS/MS data analysis was performed according to PatternLab for Proteomics 4.0 software (http://www.patternlabforproteomics.org) data analysis protocol. Protein identification was performed by employing a database comprising both conserved and serovar-specific proteins. The level of redundancy in the database needed to be addressed, as we departed from two genome annotations (SEn8/02 and SDu3) that should be merged to represent all the possible proteins. We proceeded as follows. First, the genomic sequences of strains SEn8/02 (BioSample accession no. SAMEA811652, SRA accession no. ERS022685) and SDu3 (BioSample accession no. SAMEA811682, SRA accession no. ERS022675) were assembled as previously described (48, 49) and annotated using RASTtk (50). All the annotated coding sequences of both strains then were assigned to clusters using these criteria: a minimum of 70% amino acid identity and 100% sequence coverage. This was performed using the OrthoMCL algorithm from the GET_HOMOLOGUES software v18042017 (51, 52). The sequences of each cluster containing two or more members were concatenated, adding an arginine residue (letter R) between each joined pair, so a chimeric sequence was generated. In this way, all the redundant sequences (proteins within the same cluster) were identified with a single fasta ID, while all the possible peptides searchable after *in silico* trypsin digestion were present in the chimeric sequence. In addition, serovar-specific proteins (i.e., those with no inferred homologs) were included in this protein database.

A target-reverse database, including the 127 most common contaminants, was generated using PatternLab's database generation tool. Thermo raw files were searched against the database using the integrated Comet search engine (v. 2015.2) with the following parameters: mass tolerance, *m/z* (ppm), 40; enzyme, trypsin; enzyme specificity, fully specific; missed cleavages, 1; variable modifications, methionine oxidation; fixed modifications, carbamido-methylation of cysteine. Peptide spectrum matches were filtered using PatternLab's Search Engine Processor (SEPro) module to achieve a list of identifications with less than 1% false discovery rate (FDR) at the protein level. Proteins exclusively detected in one serovar were pinpointed using PatternLab for Proteomics Approximately Area Proportional Venn

Diagram module. Statistical validation of the exclusively detected proteins relies in the Bayesian model integrated into the Venn diagram module that considers quantitative data and the number of replicates in which each protein is detected to assign *P* values and filter those proteins that are likely to be exclusively detected (53).

PatternLab's TFold module was used to identify proteins detected in both serovars but having a statistically differential abundance according to their spectral counts. TFold module relies on the Benjamini-Hochberg theoretical FDR estimator to maximize the number of identifications that satisfy a fold change cutoff that varies with the *t* test *P* value as a power law. Proteins present in at least four replicates in both serovars were considered. PatternLab relies on a stringency criterion that aims to filter out lowly abundant proteins that could produce false positives. Additionally, statistically differential proteins ($P < 0.05$) with a fold change of <1.5 were disregarded.

Cases were found of proteins expressed in both serovars but annotated in only one of them. To include these proteins in the analysis, TBLASTN (54, 55) was employed to define putative homologs of them. Best hits were required to present at least 90% sequence identity and 90% coverage to be annotated as putative homologs.

Functional enrichment analysis of differentially represented proteins between both serovars. Functional enrichment analysis was performed to determine if exclusively detected and overexpressed proteins in each serovar are associated with particular biological processes, molecular functions, or cellular components.

Briefly, proteins were assigned to gene ontology (GO) terms (56, 57), and overrepresented proteins in each serovar (Table S1) were subjected to statistical analysis to determine enriched GO terms compared to all detected proteins in the serovar (Table S1). In each case, all detected proteins in at least two replicates of the serovar were employed as the background group of the analysis.

GO term annotation was done by running the eggNOG-mapper tool v0.12.7 (58) with default parameters. The DIAMOND algorithm (59) was used to infer homology between queries and sequences in the eggNOG database (60). Terms were derived from the hit in the database with the most significant E value. The use of nonexperimental data was allowed in order to get as much ontological annotation as possible. Fisher's exact test was applied for enrichment analysis as implemented in the topGO R library v1.0 (61). The test was performed with the "weight01" algorithm option, which takes into account the hierarchical structure of GO terms to avoid false positives arising from it. A *P* value threshold of 0.05 was employed to determine GO terms considered enriched among the group of interest with respect to the background.

Quantitative PCR (RT-qPCR). For bacterial mRNA quantifications, strains SDu1, SDu3, SDu5, and SDu6 from serovar Dublin and SEn31/88, SEn8/89, SEn251/01, and SEn8/02 from serovar Enteritidis were grown to exponential phase under GMC or routine optimal growth conditions (LB, 37°C, aerobically at 200 rpm), and total RNA was extracted using an RNeasy minikit (Qiagen), with a pretreatment using RNAprotect bacterial reagent (Qiagen), and then 0.5 μg of this RNA was treated with DNase (Invitrogen) and reverse transcribed using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) and random primers in a final 20-μl reaction mixture. For real-time PCR, 2 μl of a 1/16 dilution of the resulting cDNA was used as the template using Sybr green (QuantiTect; Qiagen) in a Corbett RotorGene 6000 or ABI 7900 HT (Applied Biosystems) thermocycler. Primer sequences used are shown in Table S4. The cycling program was 15 min at 95°C and 40 cycles of 15 s at 95°C, 1 min at 60°C, and a dissociation curve increasing 1°C every 5 s until reaching a temperature of 95°C. For the analysis, we used the comparative threshold cycle (C_T) method for relative mRNA quantitation (62), using *icdA* as the normalizing gene and an arbitrarily selected strain (SEn8/02) as the calibration condition. No differences in *icdA* mRNA levels were found between strains of both serovars grown under GMC or LB conditions. Each isolate was assayed in triplicate. Non-reverse-transcribed controls rendered no detectable C_T values or were amplified at least 5 cycles later than the corresponding reverse-transcribed samples.

Construction of deletion mutants. Deletion of *dps*, *yciF*, and *ygaU* genes from the chromosome of *S.* Enteritidis (strain SEn 8/02) and *S.* Dublin (strain SDu3) was performed using a standard Lambda Red recombinase system with pKD46, pKD3, or pKD4 template plasmid as described previously (63). First, the chloramphenicol and kanamycin resistance cassettes were PCR amplified from pKD3 and pKD4, respectively, using primers P1 and P2 (Table S4). *dps* then was replaced by the chloramphenicol cassette in the chromosome of both isolates by electroporation of previously pKD46-transformed cells with PCR products obtained using hybrid primers indicated in Table S4 and the corresponding resistance cassette as the template. Thus, SEn8/02 *dps*::*cat* and SDu3 *dps*::*cat* mutant strains were obtained. In the same way, *yciF* and *ygaU* were replaced by the kanamycin cassette in both isolates; therefore, SEn8/02 *yciF*::*kan*, SDu3 *yciF*::*kan*, SEn8/02 *ygaU*::*kan*, and SDU3 *ygaU*::*kan* strains were constructed. The deletion mutations were transduced into fresh genetic backgrounds (SEn8/02 and SDu3) using bacteriophage P22. The absence of all replaced genes was confirmed by three PCRs using the respective common test primers c1/c2 or k1/k2 and nearby locus-specific primers (Table S4), as recommended by the Datsenko and Wanner protocol (63). For double mutant construction, P22 transduction of a single mutation into a recipient strain harboring the other mutation was performed, provided each gene was inactivated with a different antibiotic resistance cassette. Growth rate in LB, motility, and agglutination capacity with somatic antisera (anti-O9) were evaluated to discard alterations in the mutant strains compared to parental strains.

Oxidative, acid, and osmotic stress resistance assays. Oxidative stress assays with hydrogen peroxide were performed as described by Halsey et al. (32), with slight modifications. Briefly, overnight cultures of mutants and the respective parental strains were grown in LB broth and diluted to a

concentration of 1×10^6 CFU/ml in an oxidative solution prepared by adding H_2O_2 to sterile PBS to a final concentration of 0.5 mM. Aliquots were removed for dilution at time zero and after 2 h of incubation at 37°C statically. The number of viable cells was determined by serial 10-fold dilutions in PBS and plating on LB agar. The survival percentage was calculated for each strain as the percentage of CFU obtained at 2 h with respect to the number of CFU obtained at time zero. As a control, each strain was incubated in PBS alone, and the numbers of CFU obtained after 2 h of incubation were similar to those at time zero (data not shown).

For acidic stress resistance assays, HCl was used to adjust the pH of LB to 3.1; this was then used as the acidified growth medium. Overnight bacterial cultures grown in LB for 14 to 16 h were diluted 1/10 in this medium and incubated at 37°C, nonagitated. Aliquots were removed at time zero and after 1, 2, 3, and 6 h of incubation. The appropriate dilution of cultures in PBS was selected and plated on LB agar for CFU counting. Percent survival following acidic challenge was obtained for each strain by calculating the percentage of CFU at the selected times to the number of CFU at time zero.

Resistance to osmotic stress by sodium chloride (NaCl) was evaluated. Briefly, overnight cultures of mutants and the respective parental strains were grown in LB broth. Serial dilutions then were plated in LB agar plates supplemented with a final concentration of 0.8 M NaCl (10-μl drops in duplicate) and incubated at 37°C for 24 h. Differences in survival between the mutants and the respective parental strains were evaluated by comparing the number of CFU and macroscopic aspect of the colonies at the selected time.

Animal experiments. SDu3, SEn8/02, and the respective single and double mutants (Table 4) were grown overnight at 37°C at 200 rpm in LB broth, diluted 1:20 in fresh medium, cultured under the same conditions until an OD_{600} of ~1.2 to 1.3, and diluted 1/100 in sterile PBS. Groups of five 6- to 8-week-old female BALB/c mice (provided by the National Division of Veterinary Laboratories, Uruguay) were infected intragastrically with 2×10^6 to 5×10^6 CFU/animal (1:1 ratio of wild-type [WT] and mutant) of the indicated bacterial strain. Appropriate dilutions of bacterial inocula were plated on LB agar with and without the corresponding antibiotic for CFU counting. At 4 days p.i., mice were sacrificed by cervical dislocation. Bacterial loads in spleens were analyzed by homogenizing the organ in sterile PBS and plating appropriate dilutions on LB agar plates. At least 100 colonies per organ were replica plated onto LB agar containing chloramphenicol or kanamycin as appropriate and onto LB agar with no antibiotics. The competitive index (CI) was calculated based on the ratio of the mutant/WT from the spleen homogenate in relation to the mutant/WT ratio in the inoculum. A CI of 1 indicates that the virulence of the tested strains is equal. A CI of <1 shows that the mutant is less virulent than the WT, whereas a CI of >1 indicates that the mutant is more virulent than the WT.

Experiments with animals were performed according to national guidelines for animal experimentation that meet the International Guiding Principles for Biomedical Research involving animals, and all protocols were approved by the University Ethics Committee.

Statistical analysis. For the analysis of differences in the mRNA levels and resistance to stress conditions between strains, the Mann-Whitney test (GraphPad Prism software 8.4.1) was used, considering the difference statistically significant if the *P* value was <0.05. In animal experiments, to evaluate if the CI was significantly different from 1, we used a one-sample, two-tailed *t* test (GraphPad Prism software 8.4.1), considering the difference statistically significant if the *P* value was <0.05.

Data availability. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (64) partner repository with the data set identifier PXD021353.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.2 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.01 MB.

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A.Y.M.-S., B.D., G.M.T., A.M., and L.Y. conducted assays and discussed results. M.L. performed bioinformatic analyses. R.D. and J.A.C. discussed the results and revised the manuscript. A.Y.M.-S., L.B., and L.Y. conceived the experimental design and interpreted and discussed the results. L.Y. conceived the manuscript, and all the authors contributed to writing and making figures.

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