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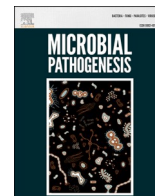
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## The global transcriptomes of *Salmonella enterica* serovars Gallinarum, Dublin and Enteritidis in the avian host

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### ABSTRACT

*Salmonella enterica* serovar Gallinarum causes Fowl Typhoid in poultry, and it is host specific to avian species. The reasons why *S. Gallinarum* is restricted to avians, and at the same time predominately cause systemic infections in these hosts, are unknown. In the current study, we developed a surgical approach to study gene expression inside the peritoneal cavity of hens to shed light on this. Strains of the host specific *S. Gallinarum*, the cattle-adapted *S. Dublin* and the broad host range serovar, *S. Enteritidis*, were enclosed in semi-permeable tubes and surgically placed for 4 h in the peritoneal cavity of hens and for control in a minimal medium at 41.2 °C. Global gene-expression under these conditions was compared between serovars using tiled-micro arrays with probes representing the genome of *S. Typhimurium*, *S. Dublin* and *S. Gallinarum*. Among other genes, genes of SPI-13, SPI-14 and the macrophage survival gene *mig-14* were specifically up-regulated in the host specific serovar, *S. Gallinarum*, and further studies into the role of these genes in host specific infection are highly indicated. Analysis of pathways and GO-terms, which were enriched in the host specific *S. Gallinarum* without being enriched in the two other serovars indicated that host specificity was characterized by a metabolic fine-tuning as well as unique expression of virulence associated pathways. The cattle adapted serovar *S. Dublin* differed from the two other serovars by a lack of up-regulation of genes encoded in the virulence associated pathogenicity island 2, and this may explain the inability of this serovar to cause disease in poultry.

### 1. Introduction

*Salmonella enterica* is an important pathogen in both humans and animals [1,2]. It is a broad species divided into six subspecies and more than 2600 serovars [3], most of which cause self-limiting diarrhoea and have a broad host range. Some serovars, such as *S. Typhi*, *S. Choleraesuis*, *S. Dublin*, and *S. Gallinarum*, however, cause severe systemic diseases, and they only infect one or a few host-species. They are therefore termed host specific or host adapted serovars, depending on the number of hosts they infect [4]. Even though pieces of information

have been assembled along the way (see Refs. [4–6] for reviews of the literature), the underlying mechanism(s) behind the adaptation to only one or a few hosts, and the reason that these serovars have such a high tendency to cause systemic infection remain unknown.

Studies in sheep, pigs, cattle, and hens have shown that host specific serovars are not superior at invading the intestine of the preferred host compared to other *Salmonella* serovars [7–10]. Host specificity mechanisms are therefore most likely expressed at systemic sites, either in phagocytic cells in the lamina propria of the intestine, or in the spleen and liver of the preferred host [4,11,12]. A way to increase

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understanding of host pathogen interaction is to study the regulation of genes in response to contact with the host. This method has been used to identify genes involved in survival and propagation of the broad host range *S. Typhimurium* inside cultured epithelial and macrophage cell lines [13,14], and to determine how macrophages react to infection with host specific and broad host range *Salmonella* serovars [15,16]. However, relying only on cell lines as a model to understand *Salmonella* pathogenicity may be misleading [17], and there is a need to establish approaches, which allow evaluation of the global gene response in *Salmonella* serovars during infection of the host. Comparison of gene responses between host specific and broad host range serovars in the same host may increase our understanding of what are the characteristics of an infection caused by a host specific serovar in its preferred host.

The three serovars *S. Gallinarum*, *S. Dublin* and *S. Enteritidis* are phylogenetically closely related within group-D *Salmonella* [18,19], but they have very different host range. *S. Gallinarum* is host restricted to poultry, where it causes Fowl Typhoid [4]. *S. Dublin* is adapted to the bovine host. It can cause systemic infections in other hosts including mice and humans [20], and it invades in the intestine of chickens to the same degree as *S. Gallinarum* and *S. Enteritidis* [9], but it never causes pathology in this host. *S. Enteritidis* is a broad host serovar causing enteritis in many different hosts including humans. It is mostly associated with self-limiting gastro enteritis [21], but of relevance to the current study, it may occasionally cause systemic infection in young chicken [22]. The close genetic relationship between these three serovars, coupled with the differences in host range and differences in the tendency to cause systemic disease in the avian host, make them an ideal study object to understand how gene-regulation during infection differ between host-restricted and non-host restricted serovars.

The aim of this study was to develop a methodological approach to study gene expression during *Salmonella* infection in the hen, and to use this approach to identify genes that are significantly regulated in *S. Gallinarum* (the host specific serotype), compared to *S. Dublin* (adapted to cattle) and *S. Enteritidis* (broad host range) when infecting the hen.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

*S. Gallinarum* G9, *S. Dublin* 2229, and *S. Enteritidis* P132344 [23–25] were used in this study. They were grown in Luria Bertani (LB) medium (Becton, Dickinson and Company, Denmark) at 37 °C or on solid agar (LB broth with 1.5% agar) at 37 °C or 41 °C (*S. Gallinarum*).

### 2.2. Method for measurement of gene-expression in vivo

#### 2.2.1. Encapsulating bacteria in dialysis tubes

A dialyse tube with a diameter of 6.4 mm and a MWCO (Molecular Weight Cut Off) of 100,000 Da (Spectra/Por® Biotech Cellulose Ester (CE) Dialysis Membranes, Spectrum Laboratories) was tied up in one end using Dermafil green polyester surgery suture (Kruuse, Denmark). The three *Salmonella* strains were grown to stationary phase in LB overnight, spun down, and resuspended in 1 ml Dulbecco's phosphate-buffered saline (DPBS) without magnesium and calcium (Gibco®, Invitrogen), resulting in a suspension with approximately  $10^{10}$  bacteria/ml. The solution was transferred to the dialyse tube. The tube was closed with sutures, and the resulting sac was either placed in the abdominal cavity of a chicken, as described below, or placed in a reference medium. This consisted of DPBS (KCl (0.2 mg/ml),  $\text{KH}_2\text{PO}_4$  (0.2 mg/ml), NaCl (8 mg/ml) and  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  (2.160 mg/ml)) (Gibco®, Invitrogen) supplemented with  $\text{NH}_4\text{Cl}$  (1 mg/ml),  $\text{CaCl}_2$  (1.110 µg/ml),  $\text{MgSO}_4$  (0.241 mg/ml), glucose (0.1%), and amino acids to compensate for auxotroph phenotypes (thiamine (0.2 µg/ml), nicotinic acid (0.2 µg/ml), leucine (25 µg/ml), cystine (5 µg/ml) and aspartic acid (5 µg/ml) (Sigma, Aldrich)). The tubes in media were incubated at 41.2 °C without shaking. The amounts of bacteria transferred to the tubes were

determined by plating 10-fold serial dilutions in PBS on LB media. For each serovar, a total of four dialyse tubes were placed in separate glass tubes with minimal medium and four dialyse tubes were incubated in the peritoneal cavity of hens, with two tubes in each hen containing the same serovar to prevent that essential factors produced or induced in the host by one serovar could cross-feed to other serovars.

#### 2.2.2. Surgical infection procedure

Nine-to eleven-week-old female Lohmann LB-Lite chickens from a flock that adhered to the Danish *Salmonella* control program, and which was free from *Salmonella* as measured both by bacteriological and serological techniques, were anesthetized with Isofluran (Pharmacia) continuously supplied through a mask at a level of 1.5–3.0% in an oxygen flow of 1.5–2.0 L/min. Feathers were removed from the abdominal area followed by disinfection of the skin with 70% ethanol. After opening the abdomen with an approximately two cm transverse incision, dialyse tubes were placed in the abdominal cavity, which was closed with 3–4 stitches and the chicken was brought out of anaesthesia. After 4 h, the hen was re-anesthetized, the abdomen was opened, and dialyse tubes were removed from the abdominal cavity (Fig. S1). The chicken was euthanized by decapitation while it was still anesthetized. Surgical procedures were carried out with permission from the Danish Experimental Animal Inspectorate, permission no. 2009/561–1675.

### 2.3. RNA extraction and purification

After incubation in either the reference medium or inside the chicken, dialyse tubes were quickly flushed with DPBS (Gibco®, Invitrogen), opened and the content transferred to a 12-ml centrifuge tube (TPP) containing two ml RNAlater® (Ambion®). The centrifuge tube was centrifuged for 10 min at 4 °C and 10,000 rpm and the supernatant removed. RNA was extracted from the pellet using RNeasy Mini Kit (Qiagen) and FastPrep®-24 (MP Biomedicals) according to the manufacturer's instructions. To remove DNA, the samples were treated with RNase-free DNaseI (Fermentas, Thermo Scientific) following the recommendations of the suppliers. Extracted RNA was analyzed for purity and quantity with a NanoDrop 1000 Spectrophotometer (Thermo Scientific) according to the instruction from the supplier and visualized on a 1.5% agarose gel.

### 2.4. Micro-array

The microarray design was as described [26]. Briefly, it consisted of tiling DNA microarrays manufactured on  $12 \times 135\text{K}$  slides by Roche-NimbleGen (<http://www.nimblegen.com>). The array contained probes for the *S. Typhimurium* strains LT2, ATCC14028, SL1344, *S. Dublin* strain CT\_02021853 and *S. Gallinarum* strain 287/91 together with sequences for their known plasmids (not *S. Gallinarum*) (GenBank entries NC\_003197, CP001363, FQ312003, CP001144, AM933173, NC\_003277, NC\_011204, NC\_017719 and NC\_017720). For *S. Dublin* CT\_02021853, the DNA microarray consisted of 31,535 probes covering 4,505 genes. Out of 4,000 randomly picked probes, the average probe length was 60 bp and the standard deviation on 100 of these was 0.52 bp. For *S. Gallinarum* 287/91 there were 29,846 probes covering 4,264 genes. The average probe length of 4,000 probes was 60 bp with a standard deviation on 100 of these probes of 0.45 bp. In addition, 14,695 control probes were present on the DNA microarray. The average probe length and standard deviation on 200 of these probes was  $55 \pm 5.23$  bp. Since the array did not contain probes, which were specific for *S. Enteritidis*, we ensured that probes for the other serovars showed acceptable hybridization with the *S. Enteritidis* strain. We randomly picked 30 genes and used BLASTN [27] to investigate the identify and coverage of the genes on the different arrays. We found the average identity to be highest in *S. Gallinarum* ( $99.8 \pm 0.2\%$ ), second highest in *S. Dublin* ( $99.5 \pm 1.0\%$ ) and lowest in *S. Typhimurium* ( $98.7 \pm 2.7\%$ ) (data not shown). The median values for identity were 99.9% for both *S.*

Gallinarum and *S. Dublin*, while it was 99.25% for *S. Typhimurium*. We also ensured that the “Query Coverage” was acceptable to limit the risk that a low coverage would result in a weak binding to the micro-array slide. The average query coverage between *S. Enteritidis* and *S. Dublin* was  $99.8 \pm 1.2\%$ , while it was  $98.4 \pm 8.4\%$  between *S. Enteritidis* and *S. Gallinarum*, and  $99.0 \pm 3.0\%$  between *S. Enteritidis* and *S. Typhimurium*. Based on this we found it justified to score hybridization in *S. Enteritidis* on the arrays used.

### 2.5. Hybridization

Hybridization and statistical analysis of significantly regulated genes was performed as previously described [26]. Briefly, hybridization and washing were done according to the NimbleGen protocol for expression arrays, where after slides were scanned with a Roche 2- $\mu\text{m}$  scanner and the images processed with NimbleScan v.2.5. The data obtained were exported to the XYS format using NimbleScan and processed with the help of the Bioconductor suite of tools [28]. Probe-level pre-processing was performed using a similar approach to the one for Affymetrix arrays, and univariate differential expression patterns were identified with the LIMMA package and the method TREAT [29].

### 2.6. Data analysis

We report changes in gene expression of more than two-fold ( $\text{Log}_2\text{FC} > 1$ ) compared to the reference condition, and a significance level less than 0.05 on the adjusted P-value as significantly regulated similar to the approach used for study of *Salmonella* regulation inside epithelial cells [14]. Hybridization was performed to a total of 13477 genes on the arrays. The pool of genes contained copies of the same gene on different arrays due to the presence of the same gene in different strains, and hybridization signals were scored for all strains on all arrays, allowing for internal analysis of the repeatability of the scoring of genes. If a particular gene showed upregulation/downregulation on the array with probes derived from the serovar in case (array with probes derived from the genome sequence of *S. Gallinarum* for scoring of *S. Gallinarum* for example), or was positive on two other arrays, the gene was scored as significantly regulated. However, this was without showing the opposite regulation on any array. This analysis was applied in the single gene analysis part. In analysis of pathway enrichment, genes which were significantly regulated on any array, and which were not differently regulated on another array, were scored as regulated. In this part, we further included the full information obtained on all arrays (i.e each gene was associated with a number of significant scores (here termed number of hits). By this approach, we encompassed the confidence of the hybridization results into the analysis.

### 2.7. Gene annotation and grouping

Previously published information on gene functions for the genes of interest were collected from the databases UniProtKB [30], EggNOG 5.0 [31,32], Kyoto Encyclopedia of Genes and Genomes (KEGG) [33,34], BioCyc [35], and Gene Ontology (GO) knowledge base [36–38]. Naming of genes was according to the *Salmonella enterica* Pan-genome dataset in the Biocyc database [35], and locus numbers for homologous genes across serovars was manually obtained from this database. The genes of interest were categorized into different groups based on functional classification schemes in KEGG pathways [33,34] and GO terms [38], and ancestor charts were drawn for GO terms using QuickGO [39]. STRING analysis was used to visualize interaction between significantly regulated genes [40]. Enrichment analysis for up-regulated and down-regulated KEGG pathways/GO terms were performed using online tools included with the databases. The rich factors were calculated for significantly enriched pathways through Fisher’s test ( $p$  value  $\leq 0.05$ ). In the analysis where enrichment was based on number of hits, we corrected for the fact that each gene was counted several times.

Uniquely regulated genes in *S. Gallinarum* in the hen was mapped against the list of conserved pseudogenes in *S. Gallinarum* ( $n = 151$ ), *S. Dublin* ( $n = 95$ ) and *S. Enteritidis* ( $n = 3$ ) [41].

### 2.8. Statistics

The obtained CFU data were subjected to statistical analysis to test for differences between input and output concentrations in the dialyze tubes using the Microsoft Excel 2010 Analysis ToolPak to test for differences in variance with a F-test, followed by a two-tailed  $t$ -test.

## 3. Results

### 3.1. A method for measuring gene expression of *Salmonella* in the hen

It is challenging to measure gene expression of bacteria inside an animal host because host mRNA is in vast excess of bacterial mRNA. To overcome this problem, we developed a surgery-based method to study bacteria inside the host. We placed sacs of dialysis tubes containing approximately  $10^{10}$  CFU of *S. Gallinarum* G9 or *S. Dublin* 2229 or *S. Enteritidis* P132344 in the peritoneal cavity of hens (Fig. S1). The pore-size of the dialysis tubes prevented bacteria from leaking out of the tubes, and at the same time, it allowed molecules with MW below 100,000 Da to enter the tube and influence gene expression of the *Salmonella* strains. After 4 h in the peritoneal cavity, tubes were removed, and cDNA of bacterial mRNA was used to generate gene expression profiles by micro-array analysis. CFU of the bacteria in tubes before they were placed in the hen and after 4 h in the peritoneal cavity showed no significant differences (data not shown), and thus differences in gene expression reported below were related to adaptation to the host environment rather than to growth.

### 3.2. Number of genes regulated in the three *Salmonella* serovars during infection of the hen

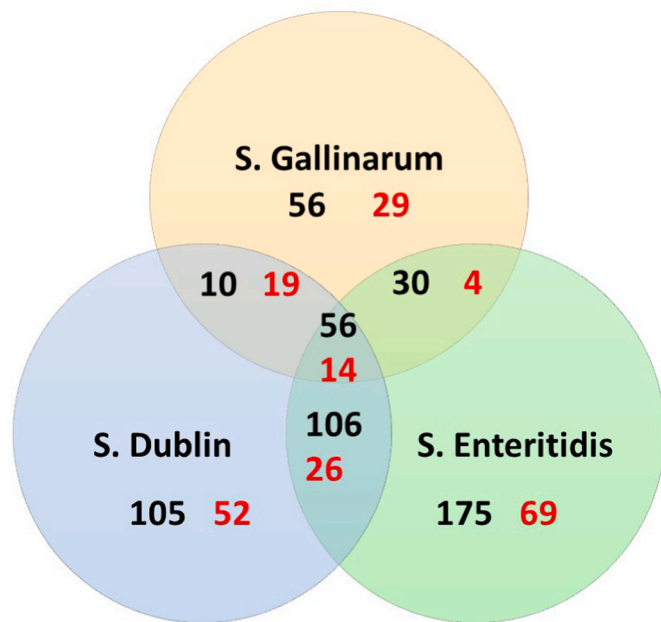
Expression of genes in the strains of the three serovars inside the hen was measured on three different tiled micro-arrays compared to expression of genes in the same bacteria placed in dialysis tubes and as pairwise comparison of expression intensities for the same gene between the bacteria harvested from the sacs in the hen. As shown in Fig. S2, a high correlation was observed between signals on different arrays.

Seven-hundred and forty-six genes were significantly regulated in at least one serovar compared to expression in minimal medium in the laboratory (Table S1). The number of unique and shared genes in the three serovars is shown as Venn diagram in Fig. 1. Fifty-six genes were uniquely up-regulated and 29 genes uniquely down-regulated in the avian adapted serovar *S. Gallinarum*.

Fifty-six genes were up regulated in concert in the three serovars. This included metabolic genes as well as genes belonging to the *Salmonella* pathogenicity island 1 regulon (*invF*, *sopE2*, *spaO*) and the *Salmonella* pathogenicity island 2 regulon (*ssaV* and *ssaN*). Only 14 genes were down-regulated in concert, and these mainly encoded metabolic enzymes, but also included the virulence genes *mgtA* and *mgtB* of SPI-3 and the gene for the heat shock sigma factor RpoH (Table S1).

### 3.3. Uniquely regulated genes in *S. Gallinarum* in the avian host

The main aim was to identify genes which were uniquely regulated during infection in the host specific serovar, *S. Gallinarum*. The up-regulated genes contained several groups of genes, which were functionally and/or spatially related to each other. This included SG3011-SG3014 of SPI-13 and four genes (SG0835, SG0837, SG0838 and SG0839) of SPI-14. Further, several genes of the SPI-1 and SPI-2 encoded Type three secretion systems (T3SSs) and their associated effector molecules (*orgA*, *orgC*, *sicA*, *sipB* and *sopB* of T3SS-1 and *ssaQ* and *ssaM* of T3SS-2) were uniquely up-regulated in *S. Gallinarum*, just as several



**Fig. 1.** Number of significantly regulated genes in *Salmonella* Gallinarum, *S. Dublin* and *S. Enteritidis* in the peritoneal cavity of hens compared to expression in a reference medium in the laboratory at 41.2 °C. Numbers shown in black are the number of significantly up-regulated genes, and numbers shown in red are the numbers of significantly down-regulated genes.

genes among ascorbate uptake and metabolism genes (*ulaA*, *ulaB*, *ulaF* and *ulaG*) were up-regulated in concert (Table 1 and Table S1).

The list of up-regulated genes contained two regulators; *sprB* is encoded within SPI-1 and regulates transcription of SPI-4 genes [42], and *fadR* is a fatty acid biosynthesis regulator [43]. In addition, the *phoP*-regulated gene *mig-14* is annotated as a putative transcription activator, and SG3014 of SPI-13, which was the gene showing the highest fold change among the uniquely regulated genes, is annotated as

**Table 1**

Top twenty uniquely up-regulated genes in the host specific serovar *S. Gallinarum* inside the hen, compared to the reference condition.

Gene name	Locus tag	Function	Log <sub>2</sub> FC			Adjusted p-value		
			SGal	SDu	SEnt	SGal	SDu	SEnt
<i>none</i>	SG3014	HpcH/Hpal aldolase/citrate lyase (SPI-13)	4.28	-1.17	0.45	<0.001	1.000	1.000
<i>none</i>	SG1870	outer membrane protein (TRL-like family)	4.13	1.81	0.52	<0.001	0.267	1.000
<i>none</i>	SG3011	macrophage colonization factor (SPI-13)	3.85	1.38	0.79	<0.001	0.517	1.000
<i>fixC</i>	SG0839	oxidoreductase (electron transfer) (SPI-14)	3.26	-0.14	0.04	<0.001	1.000	1.000
<i>ssaQ</i>	SG1698	type III secretion apparatus protein (T3SS-2)	3.21	1.66	1.51	<0.001	0.314	0.512
<i>ulaG</i>	SG4224	l-ascorbate 6-phosphate lactonase	3.19	-0.14	0.91	<0.001	1.000	1.000
<i>fadR</i>	SG1499	fatty acid metabolism regulatory protein	2.94	-0.52	0.00	<0.001	1.000	1.000
None	SG1251	inner membrane protein	2.90	-0.47	0.88	0.001	1.000	1.000
<i>sicA</i>	SG2788	chaperone protein	2.89	1.46	0.56	0.003	0.724	1.000
<i>none</i>	SG0838	acyl-CoA dehydrogenase (SPI-14)	2.82	0.27	0.71	<0.010	1.000	1.000
<i>ulaF</i>	SG4230	l-ribulose-5-phosphate 4-epimerase UlaF	2.82	0.42	1.55	0.001	1.000	0.388
<i>ulaA</i>	SG4225	PTS system ascorbate-specific transporter subunit IIC	2.79	0.15	0.02	0.001	1.000	1.000
<i>gcvT</i>	SG2950	glycine cleavage system aminomethyltransferase T	2.57	1.77	1.71	0.006	0.220	0.262
<i>fdx</i>	SG2573	electron carrier protein (ferridoxin sulfur ion binding)	2.55	1.32	1.66	0.002	0.799	0.204
None	SG3013	monoamine oxidase (SPI-13)	2.53	0.90	1.71	<0.001	1.000	0.098
<i>mraY</i>	SG0126	phospho-N-acetylmuramoyl-pentapeptide- transferase	2.53	1.10	0.32	0.001	1.000	1.000
<i>nuoM</i>	SG2346	NADH dehydrogenase I chain M	2.52	-0.10	0.33	<0.001	1.000	1.000
None	SG3012	Putative hydrolase (SPI-13)	2.51	-0.26	0.85	>0.001	1.000	1.000
None	SG0837	electron transfer flavoprotein subunit alpha (SPI-14)	2.50	-0.53	0.99	<0.001	1.000	1.000
<i>orgA</i>	SG2773	oxygen-regulated invasion protein OrgA (T3SS-1)	2.49	1.14	0.89	0.003	1.000	1.000
<i>dcd</i>	SG2153	deoxycytidine triphosphate deaminase	2.46	1.64	0.00	<0.001	0.091	1.000
<i>orgC</i>	SG2771	cytoplasmic protein (T3SS-1)	2.44	1.45	1.79	0.004	0.538	0.118
<i>mig-14</i>	SG2687	Mig-14 transcriptional activator	2.43	0.77	0.29	0.027	1.000	1.000
<i>sprB</i>	SG2769	transcriptional regulator (SPI-1/SPI-4)	2.37	1.41	0.57	0.046	0.817	1.000
None	SG3801	aryl sulfotransferase	2.34	-0.66	0.95	0.014	1.000	1.000

SGal. *S. Gallinarum*, SEnt. *S. Enteritidis*, SDu. *S. Dublin*. Fold change is relative to expression in Dulbecco's phosphate-buffered saline (DPBS) at 41.2 °C.

a putative LysR-family transcriptional regulator. Uniquely up-regulated genes in *S. Gallinarum*, where Log<sub>2</sub>FC was less than 0.25 in the two other serovars included *fixC*, *fadR*, SG0835/SG0837/SG0838 (all SPI-14), *ulaA*, *ulaB* (both of ascorbate utilization), *nuoM*, SG3801 (SPI-13), *mreB*, *acnB*, *yeeA*, and *yhcB* (Table 1 and Table S1). STRING analysis of the uniquely up regulated genes using the STM locus tags was performed to illustrate the interaction between the gene products. This revealed five clusters encompassing virulence and metabolism genes or mixtures of these two terms (Fig. S3). This analysis showed no enrichment of KEGG or GO terms.

The uniquely down-regulated genes were dominated by genes of the arginine metabolism (*cadAB*, *artJ*, *argCDE*), components of the anaerobic nitrogen reductase (*narHIJY*), and genes of the DNA-damage response (SOS response) system (*lexA*, *sulA*, *recA*, and *recN*) (Table 2 and Table S1).

The list of uniquely down-regulated genes was also subjected to STRING analysis (Fig. S4). This revealed enrichment of KEGG pathways (5), as well as GO terms within the categories biological processes (5), molecular functions (2) and cellular components (2), primarily confirming down-regulation of genes of nitrogen and arginine metabolism and DNA-stress associated genes (Table S2).

The uniquely regulated (up and down) genes in *S. Gallinarum* only involved genes which are not conserved pseudogenes in any of the three serovars investigated.

#### 3.4. Virulence gene expression inside the hen compared to growth in minimal medium

We performed a detailed mapping of expression of known virulence genes inside the hen, and followed up with analyses of the trend of regulation for the homolog genes in the other serovars, irrespectively of whether these genes were significantly regulated (Fig. 2).

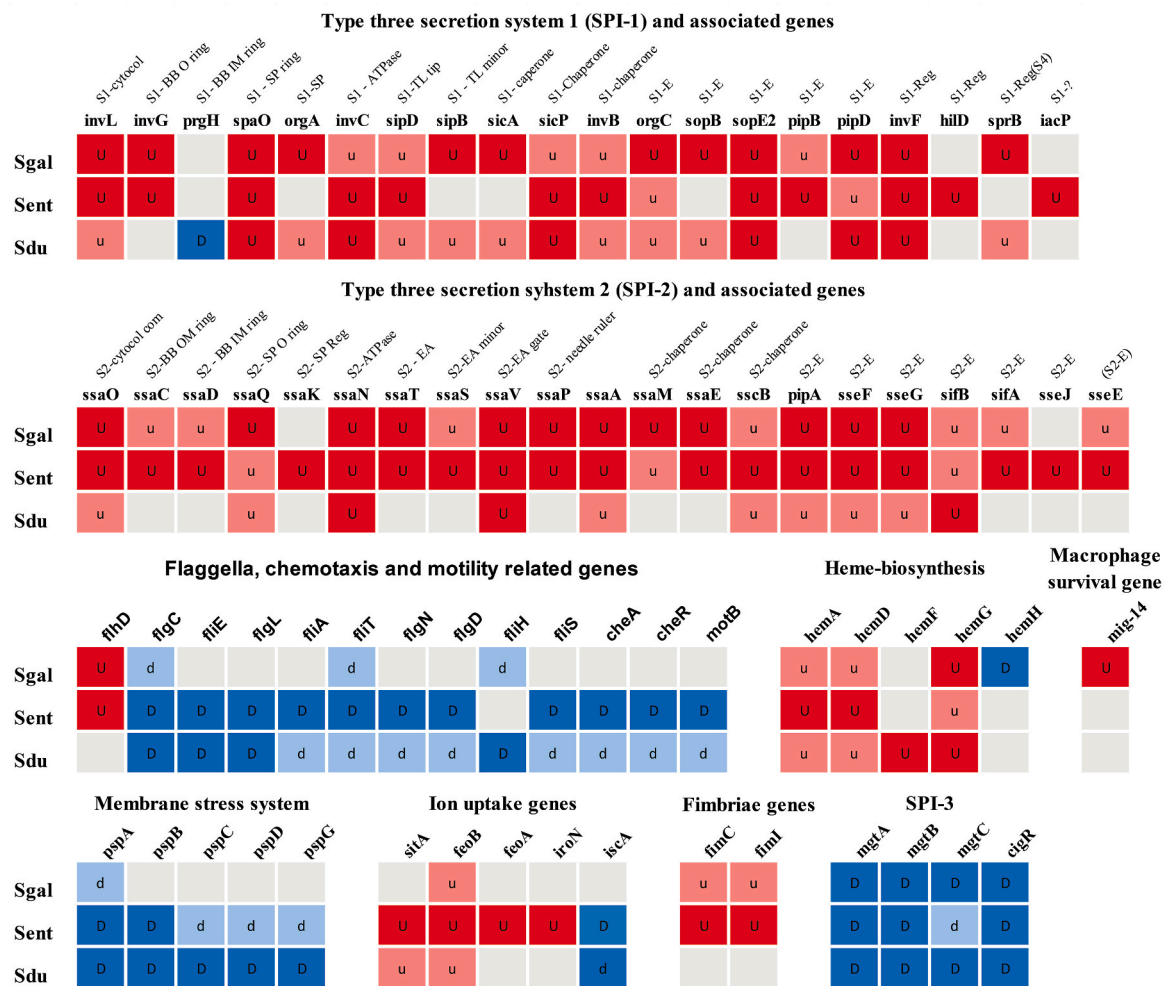
Structural and chaperone genes of the T3SS encoded in SPI-2, such as *ssaM*, *ssaE*, *ssaS*, *ssaT*, *ssaD*, and *ssaP* as well as effector molecules (*sifA*, *sseE*) were generally up-regulated or showed a trend for upregulation in *S. Gallinarum* and *S. Enteritidis* without concurrent regulation in *S. Dublin*. For SPI-1 encoded genes, the tendency was not so clear. 12 genes of T3SS-1 were significantly up-regulated in *S. Gallinarum* and *S.*



**Table 2**  
Top 10 uniquely down-regulated genes in *S. Gallinarum* in the hen compared to growth in minimal medium.

Gene name	Locus tag	Function	Log <sub>2</sub> FC			Adjusted p-value		
			SGal	SDu	Sent	SGal	SDu	SEnt
cadB	SG2595	APC family lysine/cadaverine transport protein	-5.58	-0.50	0.14	<0.001	1.000	1.000
cadA	SG2596	lysine decarboxylase1	-4.15	-0.22	-0.27	0.002	1.000	1.000
ybiJ	SG0802	periplasmic protein YbiJ	-3.92	-1.24	0.13	<0.001	0.992	1.000
artJ	SG0867	arginine ABC transporter substrate-binding protein ArtJ	-3.25	-2.42	-2.47	0.016	0.125	0.109
naY	SG1354	nitrate reductase 2 subunit beta	-3.12	-0.24	-0.62	<0.001	1.000	1.000
narI	SG1356	nitrate reductase 1 gamma subunit	-3.05	-0.13	-0.15	<0.001	1.000	1.000
argD	SG3971	bifunctional N-succinyl-diaminopimelate-aminotransferase/acetylornithine transaminase protein	-2.96	-1.88	-0.94	0.013	0.352	1.000
glpD	SG3913	glycerol-3-phosphate dehydrogenase	-2.91	-0.58	0.21	<0.001	1.000	1.000
narH	SG1549	nitrate reductase 1 beta subunit	-2.85	1.25	-0.88	<0.001	0.798	1.000
ycjX	SG1431	YCJC family	-2.72	1.42	-0.91	<0.001	0.365	1.000

Sgal: *S. Gallinarum*, Sent: *S. Enteritidis*, Sdu: *S. Dublin*. Fold change is relative to expression in Dulbecco's phosphate-buffered saline (DPBS) at 41.2 °C.



**Fig. 2.** Significantly regulated virulence genes in *S. Gallinarum* (Sgal), *S. Enteritidis* (Sent) and *S. Dublin* (Sdu) in the hen compared to growth in minimal reference medium *in vitro*. Genes shown in dark red with large U letter were significantly up-regulated, while genes shown in light red with small letter u showed the same trend (between 1 and 2 folds up-regulated) but were not significantly regulated. Genes shown in dark blue (with large D) and light blue (with small d) indicate the same situation for down-regulated genes.

Enteritidis (not the same genes), while 5 (*S. Gallinarum*) and 2 (*S. Enteritidis*) genes showed a trend for up-regulation. The corresponding number of genes in *S. Dublin* was 6 significantly regulated and 9 with a trend for up-regulation. Overall, none of the genes of T3SS-1 and T3SS-2 were uniquely regulated in the host specific *S. Gallinarum* without showing a trend for up-regulation in one or both of the two other serovars.

Genes related to motility, chemotaxis and flagella production were down-regulated or not regulated in the peritoneal cavity of the hen, with the notable exception that the major regulator of the flagella operon, *flhD*, was up-regulated in *S. Gallinarum* and *S. Enteritidis* (Fig. 2). The *mgt* locus of SPI-3 was down-regulated in all serovars, however the major macrophage survival protein of this operon, *mgtC*, was only significantly down-regulated in *S. Gallinarum* and *S. Dublin*, while it

showed a tendency for down-regulation in *S. Enteritidis* ( $\text{Log}_{10}\text{FC} -2.38$ , but  $p\text{-value} > 0.05$ ).

*S. Enteritidis* showed a higher expression of iron-binding and iron-transporting systems than *S. Gallinarum* and *S. Dublin*, and no iron-binding systems were uniquely regulated in the host specific *S. Gallinarum*. Four genes of the *hem*-operon (*hemADFG*) were upregulated or showed a trend toward upregulation in all serotypes, while *hemH* encoding the last step in the heme biosynthesis was down-regulated (Fig. 2). Only *fimC* and *fimI* of the type-1 fimbria operon were significantly regulated among fimbriae genes and only in *S. Enteritidis*. Finally, the membrane-shock-protein operon, *pspABCDG* was uniformly down-regulated in *S. Dublin*.

### 3.5. Analyzing for general patterns among significantly regulated genes in *S. Gallinarum*

In addition to single gene differences, we also analyzed data for more general trends by focusing on enrichment of KEGG pathways and GO-terms. In this analysis, we included the information on the number of hits on all three arrays for a particular gene in a particular serovar. The distribution of hits in the three serovars based on comparison to expression in the *in vitro* reference condition appears from the volcano plots in Fig. S5 (top panel). We complemented the results with an analysis of enriched pathways based on the comparison between serovars of expression of each gene inside the hen. The numbers of hits for the significantly regulated genes by this analysis, again encompassing hits on all arrays, are shown in Fig. S5, bottom panel.

Only six pathways (two up and four down) were enriched in common between the three serovars by KEGG analysis compared to the reference condition (Fig. 3). The up-regulated pathways had KEGG map-number 00195, which encompasses elements of the ATPase synthetase, and the map-number 00240, signifying that *pyr*-genes were up-regulated in all three serovars. The shared, down-regulated pathways were map-numbers 00220, 01210, 04141 and 00750, corresponding to a common down-regulation of genes of arginine and cadaverine biosynthesis, 2-oxocarboxylic acid metabolism, protein procession, and the KEGG term for genes involved in Vitamin B metabolism. The genes associated with these enriched pathways are listed in Table S3.

*S. Gallinarum* showed enrichment of 32 pathways, 18 of which contained up-regulated genes and 14 down-regulated ones. The corresponding numbers of enriched pathways for *S. Enteritidis* and *S. Dublin* were 43 and 50 (Fig. 3). Nine up-regulated pathways were enriched in *S. Gallinarum* without being enriched in the two other serovars, and thus constituted candidates for pathways of particular importance to the host specific serovar (Fig. 3). Eight of these were related to metabolic

processes (see Fig. S6 for graphic presentation of these pathways on a sketch of bacterial metabolism) in addition to the KEGG pathway, invasion of epithelial cells (Kegg map number 05100). Five pathways with down-regulated genes were uniquely enriched in *S. Gallinarum*, all of which were involved in metabolism (Table S3). When signal strength of each gene inside the hen was compared, 19 KEGG pathways were enriched in *S. Gallinarum*, 15 based on up-regulated genes and four contained down-regulated ones (Fig. S7). These pathways contained mainly genes involved in metabolism, distributed on lipid metabolism, amino acid metabolism, carbohydrate uptake, carbohydrate metabolism, amino sugar and nucleotide metabolism, and nitrogen metabolism. The four enriched pathways with down-regulated genes related to carbohydrate metabolism, amino acid metabolism, sulfur metabolism and carbohydrate uptake. The enrichment of the term PTS systems based on up-regulated genes was due to regulation of phosphoenolpyruvate dependent *sgaB* and *sgaT* genes, and the gene for the ascorbate transporter, *ptxA*. Contrary, the enrichment of PTS system based on down-regulated genes related to significant regulation of glucitol/sorbitol transport. Genes associated with these enriched pathways can be seen from Table S4. Notably, the enrichments of pathways, which was based on up-regulated genes and which were specific to *S. Gallinarum*, were the same in the comparisons between *S. Gallinarum* and *S. Enteritidis* and the comparison between *S. Gallinarum* and *S. Dublin* as only one enrichment was unique to each comparison.

KEGG focuses mainly on metabolic pathways, and in order to make the analysis comprehensive, analyses of enriched GO terms were performed based on the same datasets. An overview of results for *S. Gallinarum* based on comparison to growth in the reference medium is shown in Fig. 4. Sixty-one GO terms were enriched uniquely in *S. Gallinarum* (19 based on up-regulated genes and 42 based on down-regulated ones) compared to gene expression in the reference condition. The main enriched terms based on up-regulated genes were membranes in the category of cellular components, transferase activity in the category of molecular functions, and vitamin and carbohydrate derivate metabolism in the category of biological processes. Based on down-regulated genes, the respirasome was the main enriched term in cellular components, oxidoreductase activity (nitrate reductase) in cellular components, and response to abiotic stimulus and cellular response to stimulus and part of response to stimulus in biological processes (Figs. S8 and S9).

## 4. Discussion

In the current study, we developed an *in vivo* surgery based model for investigating *Salmonella* gene expression in the abdominal cavity of hen. The purpose was to identify gene regulation patterns that differed

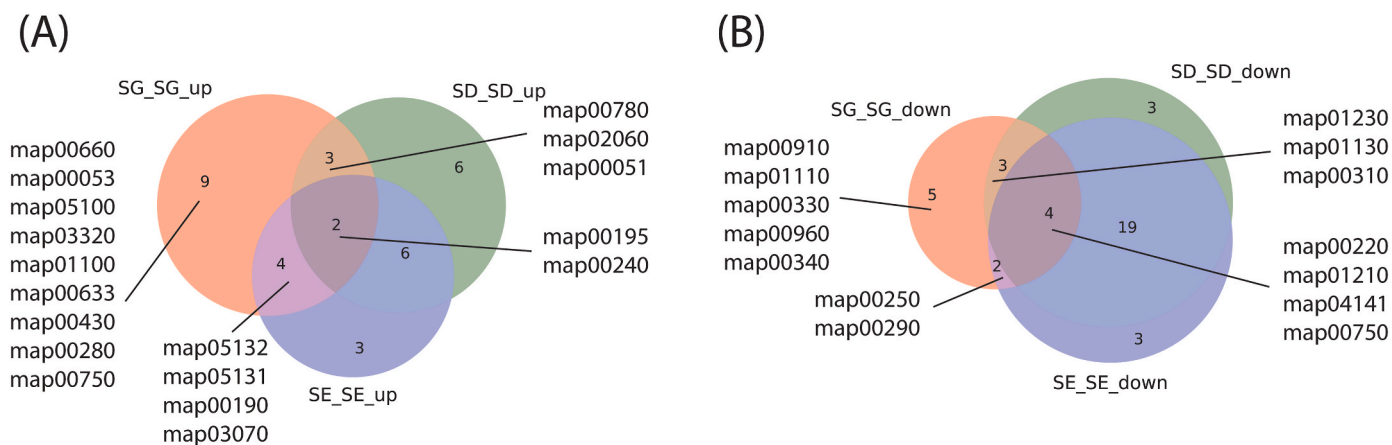
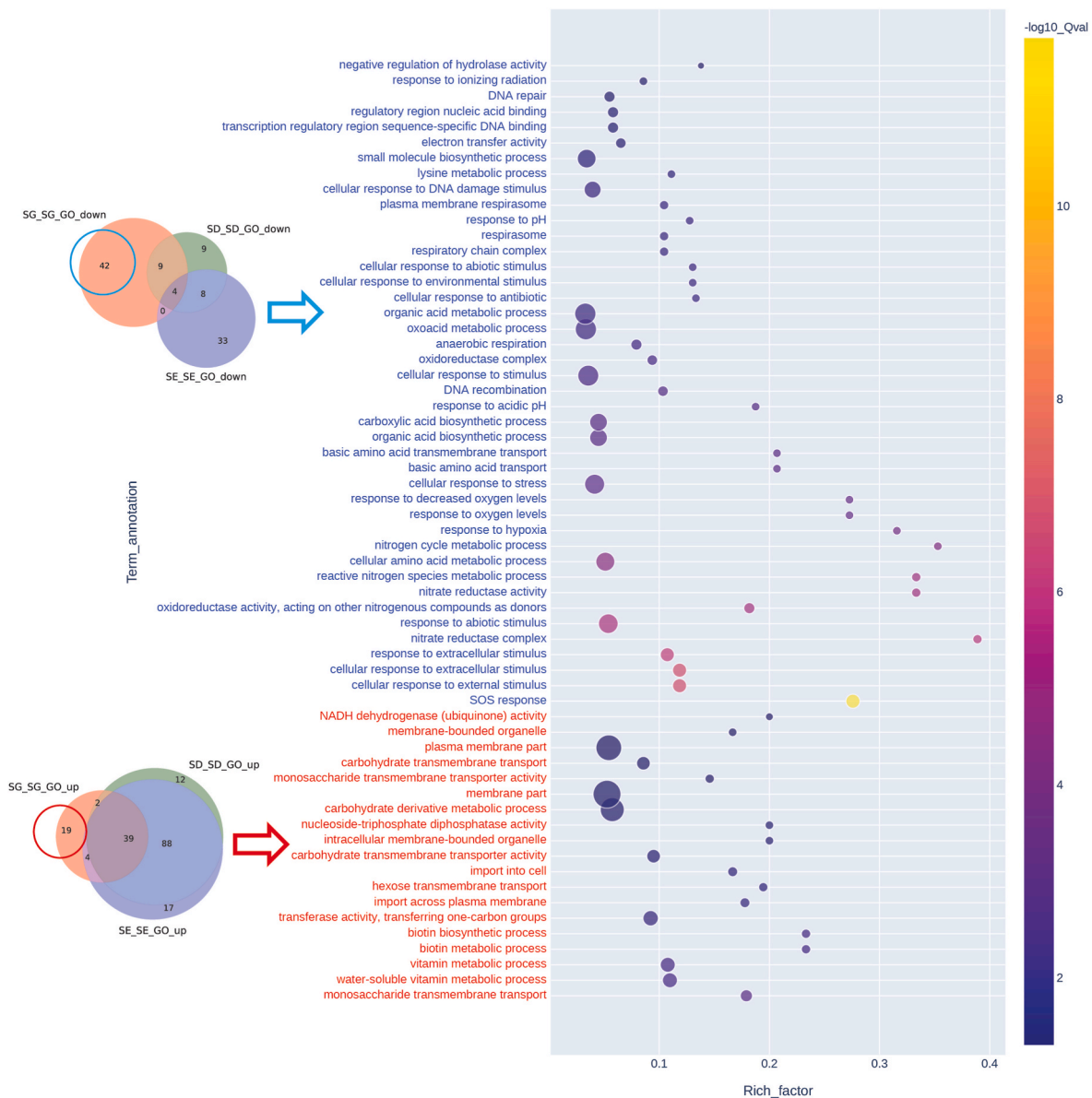


Fig. 3. Venn diagrams showing the number of enriched pathways and the corresponding KEGG map-numbers based on up-regulated (left) and down-regulated (right) genes compared to growth in minimal medium in the laboratory. The corresponding, regulated genes and the enrichment factors for each pathway are listed in Table S3.



**Fig. 4.** Enriched GO-terms for cellular components, molecular function and biological processes based on gene expression in *S. Gallinarum* during growth in the hen compared to growth in *in vitro*. The size of the dots shows the enrichment factor, and the color indicated the Q-value of the enrichment, according to the scale shown on the right side of the figure.

between *S. Gallinarum*, which is host restricted to this animal, and its close relatives within the group-D *Salmonella*, *S. Dublin*, and *S. Enteritidis*. Insight into these differences would shed light on the underlying mechanisms behind host restriction in *S. Gallinarum*, and on the reason why it has such a high ability to cause systemic infection in avian species.

Bacteria were placed in the intraperitoneal cavity, and thus the model is expected to recapitulate many of the features of a systemic infection. They were confined inside a semi-permeable sac, restricting interaction with the host to that of molecules up to 100,000 Da, and the response is likely not giving the full picture of gene expression, when *Salmonella* interacts with the host, especially due to lack of interaction with cells. For example, we consider this a likely reason that *mgtC*, which is essential for survival in the intracellular environment in many intracellular pathogens [44], was down-regulated in *S. Gallinarum* and *S. Dublin*. The lack of cell interaction is probably not only relevant in relation to interaction with phagocytic cells, but also cells of epithelium and parenchyma, since none of the many fimbria operons, except type 1

fimbriae, were induced in the model. The cutoff size for molecules to enter the sacs may also have caused a lack of interaction with important molecules of the innate humoral response to infection, such as molecules of the complement system. This restriction was necessary to make it possible to perform the global gene expression analysis, overcoming the problem of high excess of host mRNA compared to bacterial mRNA.

A full understanding of the pathogenesis would require a combined look at the bacteria and host at the same time. *S. Gallinarum* infection induced a host response in chicken derived macrophages which differs in many aspects compared to when macrophages are challenged with *S. Dublin* or the broad host range serovar *S. Typhimurium* [45]. Recently, a combination of purification techniques has enabled RNA-seq analysis of bacteria harvested from infected livers with  $>10^7$  of target organisms (*Staphylococcus aureus*) per gram tissue [46]. However, even at fulminant infection, *S. Gallinarum* does not reach such high numbers in organs [47], and *S. Dublin* could not have been used for comparison by that approach, since it does not propagate to high numbers at systemic sites in the hen [9]. In future, it could be highly interesting to include



macrophages or other cell types into the sacs in the hen to study transcriptome variation associated with the interaction of immune cells and bacteria.

The array system contained probes from three different serovars, and this increased confidence in the results. The idea was that regulation could be confirmed on different arrays, and indeed, we observed very good correlation between FCs observed for a selection of genes when measured on different arrays. Recently, a comparison of the pan-genomes of *S. Pullorum* (a biovar of *S. Gallinarum*) and *S. Enteritidis* revealed that large DNA-elements were unique to each of the serovars [48]. The array did not contain probes for *S. Enteritidis*, and there is thus a risk that we have omitted some genes, which are specific to this serovar. Since the purpose was to identify genes, which were uniquely expressed in *S. Gallinarum*, this was not considered a major problem. The virulence plasmid of *S. Gallinarum* was further not included on the array. It has previously been shown that this plasmid does not play a role in host-specificity in the avian host, as the plasmids of *S. Gallinarum* and *S. Typhimurium* can be inter-changed between the two serovars without loss of host specificity and magnitude of infection [49].

Most of the uniquely, significantly regulated genes in *S. Gallinarum* were classified as metabolic genes. Thus, the differences between the host specific serovar and the two other serovars clearly contain an important element of metabolic adaptation. *S. Gallinarum* has formed pseudogenes in many genes of metabolic pathways, which presumably are of little benefit to survival and growth in the avian host and therefore under low selective pressure [41], and pseudogene formation has not only taken place in metabolic genes; for example, pseudogene formation in effector molecules of T3SS-2 have been considered important for host specificity [50]. None of the uniquely regulated genes in *S. Gallinarum* in the current study are on the list of 151 pseudogenes published for this serovar, nor on the list of 95 conserved pseudogenes in *S. Dublin* and three genes in *S. Enteritidis* [41], ruling out that expression differences in these genes is caused by pseudogene-formation in one or more serovars.

The gene, which showed the highest unique FC in *S. Gallinarum* was SG3014 of SPI-13. This gene belongs to operon-1 of the SPI-13 island [51], and most of the remaining genes of this operon were likewise up-regulated. This pathogenicity island contributes to intestinal pathogenesis in the broad host-range serovar, *S. Enteritidis*, through addition of novel metabolic capabilities (tyrosine and D-glucuronic acid degradation) [51], however, this is not relevant for systemic infection, and it is not due to genes encoded from operon1 of the island. Transposon insertions in genes of operon 1 (SG3012, SG3014 and SG3015) are associated with attenuation, most severely for SG3012 [52], but the reason for this has not been investigated. In *S. Typhimurium*, the SPI-13 gene STM3118 of operon 1 is essential for intracellular survival in cultured macrophages [53], and knock out of the island renders this serovar avirulent in a mouse model of systemic infection [54]. STM3118 (SG3012) encodes an enzyme which hydrolyses acetyl-coA to acetate, which leads to a modified peptidoglycan, which helps the bacteria to resist degradative enzymes found in macrophages. STM3019 (SG3013) encodes for a monoamine oxidase which converts aminoacetone to a peptidoglycan precursor, aiding in the same process [53]. It would be of interest to investigate whether similar mechanisms are at play in *S. Gallinarum*, and it is highly indicated to further investigate the role of SPI-13 in host specific infection of *S. Gallinarum*, and to determine why the transcriptional regulator SP3014 is induced in this serovar and not in the other two serovars.

Shah et al. observed that knock-out of two genes of another pathogenicity island, SPI-14, caused attenuation of *S. Gallinarum* in a chicken infection model [52]. This genome island is also present in *S. Enteritidis* and *S. Typhimurium* [19]. The island encodes genes annotated with metabolic functions, as well as the LysR-family regulator, *LoiA*, which is essential for intestinal pathogenicity in *S. Typhimurium*, as it controls the oxygen dependent induction of *hilA* and *hilD* [55]. The genes SG0835-SG0839 (except SG0836) of this island were uniquely regulated

in *S. Gallinarum*, suggesting that this island may play a role in the difference in pathogenicity between this serovar and *S. Enteritidis* and *S. Dublin*, where it was not regulated. The oxygen response regulator, *LoiA* (STM0859) corresponds to SG0840 in *S. Gallinarum*. This gene was not found to be regulated inside the hen. It may be that the gene is only expressed under “intestinal” conditions. This gene, together with SG0836 (annotated as a putative electron transfer flavoprotein beta subunit), which was also not regulated, were the two genes reported to be essential for infection in chicken [52]. The regulated genes in the current study are annotated as an electron transfer oxidoreductase, an acyl-CoA dehydrogenase, an electron transfer flavoprotein subunit alpha, and a CoA-ester lyase. The role of these genes in fitness in the host is not well characterized, and further studies into the role of SPI-14 in *S. Gallinarum* host specificity in the chicken is indicated.

Genes encoding uptake and metabolic enzymes for ascorbate utilization (*ula*-operon) were uniquely up-regulated in *S. Gallinarum*, and the pathway involved in this system was enriched in pathway analysis together with genes encoding the ascorbate associated PTS system, *SgaA* and *SgaB*. Thus, use of this pathway seems to characterize the host specific infections with *S. Gallinarum*. Ascorbate metabolism is not well characterized in *Salmonella*. In *E. coli*, degradation of vitamin C is possible under anaerobic but not aerobic conditions [56]. We can only speculate why *S. Gallinarum* up-regulates genes for these enzymes. The traditional perception is that this system is used by protobacteria (*E. coli*) in the intestine, which is an anaerobic environment [57], but here we observed activity in the peritoneal cavity. Peritoneal infections can be caused by both facultative and obligate anaerobic organisms [58], suggesting an environment with low oxygen tension, and this may be the reason that *S. Gallinarum* uses this system in our model. Further studies are needed to understand whether this is important for the successful outcome of host specific infection, or whether the regulation is related to the placement of sacks in the peritoneum.

Down-regulation of metabolic genes in *S. Gallinarum* did not appear to be random, as the regulated genes clustered in pathways related to arginine metabolism, nitrogen metabolism and the non-metabolic SOS system. It is not obvious why arginine biosynthesis was down regulated during systemic infection caused by *S. Gallinarum*, and it may be related to the fact that we measured gene expression relatively to a minimal growth medium. Polyamine biosynthesis is essential for Fowl Typhoid infection, and this requires synthesis based on arginine [59]. Arginine conversion to polyamines are likewise required for systemic infection with *S. Typhimurium* in mice [60], and metabolic modelling performed to identify the combination of genes (redundant metabolic genes) required for this infection also identifies genes of arginine metabolism as essential for growth in the infection situations [61]. Contrary to this, the downregulation of genes associated with the nitrate-reductase seems logical, since this enzyme is used for anaerobic respiration in the intestine [62], and *S. Gallinarum* does not depend on propagation in the intestine to the same extent as the two other serovars, and it has previously been reported that genes of SPI-1 are dispensable for Fowl Typhoid infection, while genes of SPI-2 are essential [63].

Virulence factors of *Salmonella* have been intensively researched, and information on regulations of these could inform on differences in virulence strategies used by the three serovars. In general, genes of type three secretion systems 1 and 2 and effector molecules of these systems were up-regulated, however differently in the three serovars. Characteristically, the two serovars which are capable of systemic infection in the hen, i.e., *S. Gallinarum* and *S. Enteritidis*, shared up-regulation of many genes of T3SS-2, while only few genes of these systems were upregulated in *S. Dublin*. T3SS-2 is mainly associated with intracellular survival [63], and the lack of induction of this system in *S. Dublin* may explain why this serovar does not propagate to cause systemic infection, despite invading the intestine of chicken to the same extent as the two other serotypes [9]. Two genes, *ssaQ* and *ssaM* were uniquely (significantly) regulated in *S. Gallinarum*, but they also showed a trend for up-regulation in at least one of the other serovars, and we do not find it

likely that this gene is important for host specificity. The picture with T3SS-1 was less clear. *HilD* is required for induction of the T3SS-1 [64], and *hilD* was upregulated (significantly) in *S. Enteritidis*, only. The trend (however, non-significant) was the same in the two other serovars. As for T3SS-2, *S. Dublin* seemed less capable of induction of T3SS-1 gene expression in the chicken model than the two other serovars. Taken together, the study foremost pointed to a marked difference in induction of T3SSs between the two invasive serotypes, *S. Gallinarum* and *S. Enteritidis* and *S. Dublin*, which is apathogenic to chicken. Whether minor differences in expression of effector molecules between *S. Gallinarum* and *S. Enteritidis*, especially in T3SS-2, can explain why *S. Gallinarum* has a higher tendency to cause systemic infection requires more fine-tuned methods to find out, and probably a time course study rather than a snap-shot in time, as performed in the current study.

*mig-14* was among the uniquely regulated genes in *S. Gallinarum* inside the hen. This gene assists in persistence inside activated macrophages in *S. Typhimurium* by giving resistance towards antibacterial peptides, including cathelin-related antimicrobial peptide (CRAMP) [65], and it is reasonable to assume it has a similar role in other serovars. This gene did not show a trend for up-regulation in the two other serovars. This suggests that *S. Gallinarum* either senses the general environment inside the hen differently from the two other serovars or responds differently to a common stimulus. The bio-variant Pullorum of *S. Gallinarum* is known to persist in macrophages during later phases of the infection [12], and it may be that genes like *mig-14* are crucial for this phenotype.

None of the 14 virulence regulators proposed by Yoon et al. [66] to form the network of regulator genes, which together controls virulence during systemic infection with *S. Typhimurium* in a mouse model, were found to be regulated. This can point to major differences between serovars in virulence regulation, however, it can also mean that the infection model or array technique used are not sufficiently sensitive to study expression-differences for these regulators. The uniquely regulated genes in *S. Gallinarum* only contained one known virulence regulator, *sprB*. This regulator is encoded within SPI-1 and ensures coordinated regulation between T3SS-1 and SPI-4 [67]. Despite being named for their influence on *Salmonella* intestinal infection (*siI*), SPI-4 genes (*siIA-F*) to a varying degree affect virulence in both *S. Typhimurium* and *S. Enteritidis* in a systemic mouse model after both oral and intra-peritoneal challenge [68]. Whether this is also the case in *S. Gallinarum* needs to be investigated, but none of the genes of SPI-4 were found to be significantly regulated in the current study.

Despite iron scavenging being essential for the outcome of systemic *Salmonella* infection [69], *S. Enteritidis* was the only serovar, which up-regulated iron-binding proteins (*sitA*, *iron*, *feoA* and *feoB*). This indicated that either iron mobilization is not a significant factor of host-specificity, or it may point to a limitation of the study design, as there may be no need for these systems at the early phases of infection, where there is no active growth. However, this does not explain why *S. Enteritidis* upregulated the genes. Further studies are needed to understand if this observation is caused by differences between serovars in the speed by which they upregulate such systems, or it represent serovar differences in the need to up-regulate the systems in the peritoneal cavity.

In summary, this study has developed a surgical method that allows global expression analysis of bacteria inside the hen, and we applied the method to investigate the gene expression patterns that are characteristic of the host specific serovar *S. Gallinarum* inside the hen compared to two genetically close serovars, which are not adapted to this host. The study utilized DNA-array for gene expression analysis, but RNAseq would be equally possible to use for analysis, and in future, phagocytic or other cell types could be included in the sacs containing the bacteria to enrich the information with expression brought about by cell-to-cell contact. The host specific serovar *S. Gallinarum* was found to differ from the other two serovars with respect to both metabolic and virulence gene expression, and SPI-13, SPI-14, *mig-14* and enzymes of ascorbate

metabolism were pinpointed as targets for future deeper studies in order to understand the host specific infection.

## Ethical statement

Surgical procedures were carried out with permission from the Danish Experimental Animal Inspectorate to the senior author, permission no. 2009/561–1675.

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## CRediT authorship contribution statement

**Xiao Fei:** Writing – review & editing, Writing – original draft, Validation, Investigation, Formal analysis. **Casper Schroll:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Kaisong Huang:** Investigation, Writing – review & editing. **Jens P. Christensen:** Writing – review & editing, Supervision, Methodology. **Henrik Christensen:** Writing – review & editing, Supervision, Formal analysis. **Sebastian Lemire:** Writing – review & editing, Methodology, Formal analysis. **Mogens Kilstrup:** Writing – review & editing, Methodology, Conceptualization. **Line E. Thomsen:** Writing – review & editing, Supervision, Formal analysis. **Lotte Jelsbak:** Writing – review & editing, Supervision, Investigation, Conceptualization. **John E. Olsen:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare that there are no conflicts of interest to declare.

## Data availability

Data used are all enclosed with the supplemental data

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.micpath.2023.106236>.

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