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Salmonella typhimurium and its host : host-pathogen cross-talk, immune evasion, and persistence

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chapter nine

Intracellular gene expression profiling of *Salmonella enterica* serovar Typhimurium and mutant DLG294 that is hypersusceptible to the redox cycling agent menadione

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Abstract

DLG294 is a *Salmonella enterica* serovar Typhimurium mutant that is hypersusceptible to intracellular superoxide that is highly attenuated in vivo in C3H/HeN mice and in vitro in macrophages. The altered virulence of DLG294 is not due to increased activation of the macrophage but must be due to the lack of expression of sspJ. With the study presented here, we tried to address the role of *sspJ* by checking the broad spectrum phenotypes of DLG294 and the wild-type strain using phenotype microarrays and by looking at in vivo-regulated genes of *S. enterica* serovar Typhimurium itself during infection of host cells.

In this study we show that DLG294 is more susceptible to acid, lixivium, and antibiotics that inhibit protein synthesis, suggesting that the interior defense against toxic compounds is wrongly regulated/disregulated in DLG294. Comparison of the gene expression profiles of intracellular DLG294 and wild-type 14028s revealed only a few small differences. This indicates that *sspJ* does not have an important role in regulating (virulence) gene expression but that it has a direct role in survival under stress conditions like those encountered inside the macrophage. We propose that the absence of SspJ alters the integrity of the *Salmonella* membrane leading to increased permeability and thus enhanced susceptibility to toxic compounds and antibiotics and to the attenuated in vivo phenotype of DLG294.

Introduction

Salmonellae are Gram-negative facultative intracellular pathogens that can cause a variety of diseases in animals and man, ranging from mild gastroenteritis to severe systemic infections like typhoid fever. *Salmonella enterica* serovar Typhimurium may cause gastroenteritis in man, but causes systemic infection in mice comparable to typhoid fever in man (17). *Salmonella* predominantly invades mononuclear phagocytes and is able to cause persistent infections by evasion or disturbance of the host immune system (16). Upon infection with *Salmonella*, the host will try to develop an immune response to limit bacterial growth and to eventually kill and eliminate the pathogen. Despite the presence of a multitude of antimicrobial defense mechanisms as part of the innate immune defense system in phagocytes, *Salmonella* is able to enter, survive, and even replicate within these cells. *S. enterica* serovar Typhimurium responds to the specific host environment by expressing factors that are necessary for intracellular survival and for resistance against the defense systems of the host (6, 8, 9, 16, 20), but the exact mechanisms by which it is able to survive after phagocytosis are largely unknown.

One of the major early defense mechanisms against *Salmonella* is the production of reactive oxygen intermediates (ROI), both in vitro (18) and in vivo (24, 25, 33). Since superoxide is a by-product of normal aerobic metabolism, both eukaryotic and prokaryotic cells have evolved ways to respond to superoxide stress by the activation of genes involved in a protective response (23). Several genes and systems have been described that play a role in the defense response of *S. enterica* serovar Typhimurium against ROI, such as the SoxR/S regulon, the OxyR system, *katE* encoding cytosolic catalase, and the superoxide dismutases SocCI and SodCII (reviewed in (14)). The relative importance of each of the mechanisms involved in defense against oxidative stress for *S. enterica* serovar Typhimurium intracellular survival has not been elucidated. However, the periplasmic Cu,Zn-SOD and the type III secretion system encoded by *Salmonella* pathogenicity island 2 (SPI2) have been shown to be important in this defense, as mutants deficient in one of these systems show reduced survival within macrophages (5, 10, 34).

We have recently identified a superoxide hypersusceptible *S. enterica* serovar Typhimurium *sspJ* mutant strain DLG294 that is highly attenuated in vivo in C3H/HeN mice and in vitro in macrophages (27, 32). The attenuated phenotype is related to its hypersusceptibility to superoxide since DLG294 is able to grow out as much as the wild-type strain in cells and mice that lack one of the components of the NADPH-oxidase and as a result cannot produce superoxide (32). The exact function and mechanism of action of *sspJ*, however, are still unknown. Although hypersusceptibility to superoxide could be the major cause of attenuated virulence of DLG294, it cannot be excluded that other factors might also play a role. Virulence is not determined by superoxide sensitivity sec since many menadione-susceptible mutants are not attenuated at all (28, 29, 31). These data suggest that other factors play a role.

One way to address the differences in virulence of DLG294 and the wild-type strain is to study the macrophage activation status by comparing the gene expression profiles of

infected macrophages (22) as this might give an indication on how the macrophages respond to infection with a certain pathogen. We previously addressed the question if a possible difference in the activation status of the macrophages might explain and might clarify whether attenuation of DLG294 is solely due to its hypersusceptibility to superoxide produced by the macrophages or that additional mechanisms play a role. Since we observed no or only minor differences in gene expression profiles between DLG294 or wild-type-infected RAW264.7 macrophages (30) we have performed another study in which we checked the broad spectrum phenotypes of DLG294 and the wild-type strain using phenotype microarrays and have assessed in vivo-regulated genes of *S. enterica* serovar Typhimurium during infection of host cells as described by Eriksson et al. (7).(12)

Materials and Methods

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. Bacteria were grown overnight (ON) on LB broth plates at 37°C. The day of infection, a large number of bacterial cells were resuspended in 10 ml PBS. The bacterial cells were pelleted and resuspended in 1 ml PBS. For opsonization, 10% mouse serum was added and bacteria were incubated at 37°C for 30 min.

Table 1. *S. enterica* serovar typhimurium strains used in this study

Strain	characteristics	origin or reference
14028s	wild-type	ATCC
DLG294	14028s sspJ::MudJ	(27)
SL1344	*rpsL hisG*	(13)

In vitro acid challenge. ON cultures of bacteria grown in LB medium pH7.0 were diluted 1:100 in LB medium + 0.4% glucose pH 7.0 (unadapted) of in LB medium + 0.4% glucose pH 4.5 (adapted) and were incubated at 37°C for 1 h. A sample was taken to determine the number of bacteria before challenge with acid (t=0 h) and the remaining bacteria were spun down and resuspended in LB medium + 0.4% glucose pH 3.0 and were incubated at 37°C. At 1 and 2 h after challenge, samples were taken to determine the number of bacteria that were still viable.

Cell culture and in vitro infection model. RAW264.7 cells were grown in RPMI 1640 medium supplemented with 10% FCS, 25 mM HEPES, L-Glutamine, 100 U/ml penicillin, and 50 mg/ml streptomycin. For each extraction of RNA 1×10^8 cells were seeded in four 225 cm^2 tissue culture flasks (Costar) in RPMI 1640 medium supplemented with 10% FCS, 25 mM HEPES, and L-Glutamine. Cells were infected at an MOI of 100 with bacteria that

were opsonized or non-opsonized for 1 h at 37°C and 5% CO_2 to allow uptake of the bacteria. Then extracellular bacteria were killed by adding medium containing 30 μg/ml gentamicin. After 1 h incubation at 37°C and 5% CO_2 the medium was replaced for medium containing 5 μg/ml gentamicin and were incubated for another 2 h.

Phenotype Microarray (PM). PM tests were performed in duplicate by Biolog Inc. (Hayward, California, U.S.A.) as described in (3). All PMs were incubated at 36°C in an OmniLog and monitored for color change in the wells. Kinetic data were analyzed with OmniLog-PM software. The phenotype of mutant DLG294 was compared to that of the wild-type 14028s.

RNA extraction. After 4h, the infected RAW264.7 macrophages were lysed on ice for 30 min in 0.1% SDS, 1% acidic phenol, 19% ethanol in water. Bacterial pellets were collected by centrifugation and RNA was extracted using the Promega SV total RNA purification kit. Approximately 10^8 CFU were isolated on each time point and yielded 3-5 μg RNA. Size chromatography was done with an Agilent 2100 Bioanalyser.

Microarray. DNA microarray analysis was performed as described in (4), except that the arrays were printed on Corning CMT-GAPS™-coated slides and contained 666 extra genes from *S. enterica* serovar Typhimurium DT104 and SL1344, *S. enterica* serovar enteritidis PT4, and *S. enterica* serovar Galinarum 287/91 ("Salsa" microarray, http://www.ifr.bbsrc.ac.uk/Safety/Microarrays/default.html).

Probe labeling and scanning. RNA was first reverse transcribed into cDNA and was then labeled by random priming. For labeling protocols, see http://www.ifr.bbsrc.ac.uk/Safety/Microarrays/Protocols.html. Fluorescently labeled genomic DNA was used as a reference in each experiment. After hybridization, the slides were scanned using a GenePix 4000 A scanner (Axon Instruments). The fluorescent spots and the background signals were then quantified using GenePix Pro software (Axon Instruments). All RNA samples were hybridized to microarrays in duplicate.

Data analysis. Spots that showed a reference signal lower than the background signal plus two standard deviations were excluded from the analysis. The background signal was subtracted from each spot signal and fluorescence ratios were calculated. After performing data centring, data were analyzed using Genespring™ software (Silicon Genetics) as described previously (7). Only coding regions showing at least a two-fold difference in the mutant compared to the wild-type strain were regarded as being differentially expressed. Hierarchal clustering of gene expression profiles was performed using the Pearson correlation.

Results and Discussion

In vitro phenotypes lost by DLG294 vs 14028s. In order to address the role of *sspJ* in *S. enterica* serovar Typhimurium, we compared the broad spectrum phenotypes of the *sspJ* mutant DLG294 and the wild-type strain using phenotype microarrays. In this assay, the ability of wild-type *S. enterica* serovar Typhimurium and DLG294 to grow in the presence of 2000 different nutrients, antibiotics, and toxic compounds was evaluated by determining metabolic rates. With this assay most known aspects of cell function can be monitored and the range of phenotypes include cell surface composition and transport, catabolism, biosynthesis, macromolecules, cellular machinery, respiratory functions, and stress and repair functions. Quite a few phenotypes were lost by the *sspJ* mutant compared to the wild-type strain (Table 2). DLG294 was more sensitive to an acidic or alkalic environment as its growth is impaired at pH9.5 and pH4.5 compared to that of the wild-type strain (Table 2). We have further tested the sensitivity to acid in an in vitro challenge assay in which we used logphase LB cultures of 14028s and DLG294 grown at pH 7.0 and challenged with LB medium pH 3.0. The numbers of DLG294 declined faster than those of the wild-type strain confirming the increased susceptibility to acid (Fig. 1). However, when DLG294 bacteria were allowed to adapt at intermediate pH (pH 4.5) and were then challenged with pH 3.0, DLG294 behaved like the wild-type strain (Fig. 1).

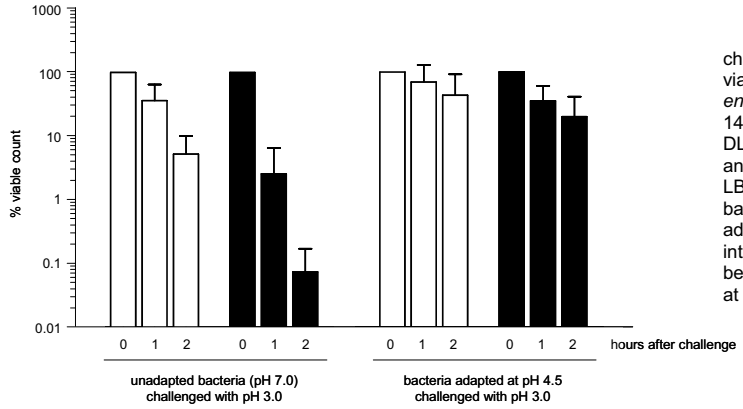

Figure 1. In vitro acid challenge assay. Percentage viable count of wild-type *S. enterica* serovar Typhimurium 14028s (white bars) and DLG294 (black bars) at 0, 1, and 2 h after challenge with LB medium pH 3.0. The bacteria were allowed to adapt in LB medium with intermediate pH 4.5 for 1 h before challenge or were left at pH 7.0 (i.e. unadapted).

DLG294 also has diminished resistance to protein synthesis inhibiting antibiotics. The macrolides inhibit bacterial protein synthesis by inhibiting the 50S ribosomal subunit. The other protein synthesis inhibitors have more diverse mechanisms of action such as inhibition of polymerization of glycoproteins (vancomycin), premature termination of chains during translation (puromycin), inhibition of translocation during protein synthesis (fusadic acid), or inhibition of peptide bound formation in the ribosomal machinery (blasticidin S). The resistance to the β-lactam type of antibiotics as well as the related cephalosporins was also impaired in the DLG294 mutant compared to the wild-type strain. These antibiotics inhibit bacterial wall synthesis and are therefore bactericidal for rapidly dividing cells.

Table 2. Phenotypes lost by DLG294 compared to wild-type 14028s

test[a]	difference[b]		mode of action
	min	max	
Ketoprofen		-70	anti-capsule, anti-inflammatory
Sanguinarine		-102	ATPase, Na^+/K^+ and Mg^{2+}
sodium pyrophosphate		-59	chelator, hydrophilic
2,2'-dipyridyl		-108	chelator, lipophilic
orphenadrine		-108	cholinergic antagonist
Prinidol		-55	cholinergic antagonist
promethazine		-76	cyclic nucleotide phosphodiesterase
9-aminoacridine		-75	DNA interchelator
4-hydroxycoumarin		-89	DNA interchelator
Acriflavine		-44	DNA interchelator
novobiocin		-87	DNA topoisomerase
domiphen bromide		-78	fungicide
D-serine		-60	inhibits 3PGA dehydogenase (L-serine and pantothenate synthesis)
trifluoperazine		-94	ion channel, Ca^{2+}
dequalinium chloride		-72	ion channel, K^+
benzothonium chloride		-100	membrane, detergent, cationic
poly-L-lysine		-110	membrane, detergent, cationic
lauryl sulfobetaine		-73	membrane, detergent, zwitterionic
amitriptyline		-95	membrane, transport
Lys-Gly		-140	N-source
Val-Lys		-81	N-source
3% urea		-86	osmotic sensitivity, urea
plumbagin		-121	Oxidizing agent
potassium superoxide		-112	Oxidizing agent
D, L-thioctic acid		-59	Oxidizing agent
cysteamine-S-phosphate		-118	P-source
pH 9.5 + amino acids[c]	-150	-66	pH, deaminase
pH 4.5 + amino acids[d]	-100	-62	pH, decarboxylase
chlorpromazine		-108	phenothiazine
compound 48/80		-53	phospholipase A, ADP ribosilation
antibiotics[e]	-223	-68	protein synthesis
respiration influencing agents[f]	-135	-55	respiration
rifamycin SV		-189	RNA polymerase
rifampicin		-112	RNA polymerase
transport influencing agents[g]		-161	transport, toxic anion
D, L-methionine hydroxamate		-64	tRNA synthetase
antibiotics[h]	-216	-66	wall, cephalosporin

[a] chemicals were tested in 96-well PMs
[b] The OmniLog-PM software generates time course curves for respiration (tetrazolium color formation) and calculates differences in the areas for mutant and control cells. The units are arbitrarily.
[c] L-phenylalanine; L-tryptophan; L-leucine; L-isoleucine; L-norvaline; glycine; L-homoserine; L-methionine; agmatine; b-hydroxy glutamate
[d] urea; D, L diaminopimelic acid; L-lysine, g-hydroxy glutamic acid; L-ornithine
[e] vancomycin; tylosin; puromycin; fusidic acid; blasticidin S; spiramycin; oleandomycin; josamysin; troleandomycin; erythromycin
[f] tetrazoleum violet; thioridazine; crystal violet; iodonitro tetrazoleum violet; sorbic acid; FCCP; sodium caprylate, cinnamic acid; CCCP; ruthenium red
[g] sodium metasilicate; sodium cyanate; chromium chloride; lead (II) nitrate; manganese (II) chloride
[h] cefoxitin; cephaloridine; piperacillin; oxacillin; nafcillin; cloxacillin; pheneticillin; aztreonam

Another major difference in phenotypes is the increased sensitivity of DLG294 to agents that influence the respiratory chain such as the respiratory uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) a drug known to inhibit the efflux systems that use the proton motive force. CCCP, however, has no effect on resistance to menadione as has been shown previously for the wild-type strain 14028s (28).

Taken together, the phenotypes lost by DLG294 could suggest that the membrane integrity of DLG294 has changed compared to the wild-type strain and, as a result, has become more leaky resulting in enhanced susceptibility to certain toxic compounds such as the macrolides, β-lactams, and cephalosporins, to stress inducing conditions such as acidic and alkalic pH, and menadione.

In vitro phenotypes gained by DLG294 vs 14028s. The phenotype microarray analysis revealed that some phenotypes were gained (Table 2) by DLG294 compared to wild-type. Since DLG294 contains a kanamycin resistance cassette in the MudJ transposon that has inserted into *sspJ*, it is more resistant to kanamycin and some other aminoglycosides (Table 3). DLG294 also has increased ability to use nitrogen-sources for growth which is consistent with the hypothesis that the membrane integrity of DLG294 has changed compared to the wild-type strain and has become more permeable for nutrients and certain toxic compounds.

Table 3. Phenotypes gained by DLG294 compared to wild-type 14028s

Test[a]	difference[b]	mode of action
L-arabinose	62	C-source
L-rhamnose	58	C-source
chloroxylenol	140	Fungicide
ethylamine	117	N-source
acetamide	103	N-source
g-D-Glu-Gly	100	N-source
Phe-Trp	83	N-source
Tyr-Ile	79	N-source
cytosine	78	N-source
b-Ala-Gly	75	N-source
b-Ala-Phe	72	N-source
D-Leu-D-Leu	56	N-source
D-Ala-Leu	55	N-source
D-lysine	55	N-source
nitrite	52	N-source
Gly-D-Val	51	N-source
adenosine	136	nutrient stimulation
2'-deoxy-adenosine	76	nutrient stimulation
thymidine-5'-monophosphate	52	P-source
phosphono acetic acid	51	P-source
kanamycin	212	protein synthesis, aminoglycoside
paromomycin	201	protein synthesis, aminoglycoside
neomycin	162	protein synthesis, aminoglycoside
geneticin (G418)	148	protein synthesis, aminoglycoside

[a] chemicals were tested in 96-well PMs
[b] The OmniLog-PM software generates time course curves for respiration (tetrazolium color formation) and calculates differences in the areas for mutant and control cells. The units are arbitrarily.

Transcriptional profiling of intracellular 14028 and SL1344. We used the recently described method for isolating *S. enterica* serovar Typhimurium RNA from intracellular bacteria (7) to compare the gene expression profiles of different strains that resided and replicated within RAW264.7 macrophages. RAW264.7 macrophages were infected as

described in Materials and Methods. At 4 h after infection, the cells were lysed and bacterial pellets were isolated and used for RNA extraction. Since the bacterial RNA was immediately stabilized, there is only minimal degradation (Fig. 2). The extracted bacterial RNA was labeled and hybridized to the *S. enterica* serovar Typhimurium "Salsa" array with labeled bacterial DNA from SL1344 grown to mid-logphase in LB medium pH 7.0 as a reference. The genes that are 2-fold differentially expressed in the intracellular bacteria compared to mid-logphase grown SL1344 in LB broth pH7.0 were equally distributed in the genome as shown for 14028s in Figure 3B. We first compared the gene expression profile of intracellular 14028s to that of SL1344 at 4 h. This was done to evaluate whether these profiles are comparable since most intracellular *S. enterica* serovar Typhimurium array data have been are generated with this SL1344 strain (7). The patterns of gene expression after normalization were only slightly different for certain genes of the 14028s strain compared to the SL1344 strain (Fig. 3C). The similarity in gene expression profiles of intracellular 14028s and SL1344 becomes even more apparent from the cluster diagram shown in Figure 3A.

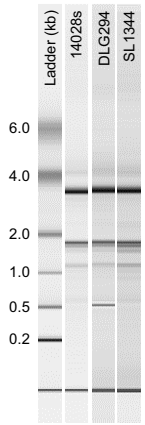

Figure 2. Size chromatographic separation of RNA. Total RNA was extracted from intracellular 14028s, DLG294 and SL1344 and analyzed on a Bioanalyser.

Direct comparison of gene expression profiles of intracellular 14028s and DLG294. We next evaluated the gene expression profile of DLG294 compared to the wild-type strain 14028s to look for genes that are differentially expressed. What becomes clear from Figure 4C is that only a few of the genes were differentially expressed and that they are located all over the *S. enterica* serovar Typhimurium genome with a few small clusters of genes showing altered gene expression. The genes that showed altered expression for DLG294 only show only a small difference in expression level as can be seen in the cluster diagram (Fig. 4B) and the relative gene expression profile of DLG294 versus the wild-type strain 14028s stays within the range of 0.3-5 fold change (Fig. 4A). For each of the genes showing altered gene expression, the relative expression was depicted in Figure 5. The expression of only 11 genes was increased for intracellular DLG294 compared to the wild-type strain and 19 genes showed decreased expression. None of the genes of the virulence gene clusters such as SPI-1 and SPI-2, nor genes encoding the superoxide dismutases were altered, indicating that the DLG294 mutant does not lack expression of the well-known virulence genes nor the defense mechanisms against superoxide.

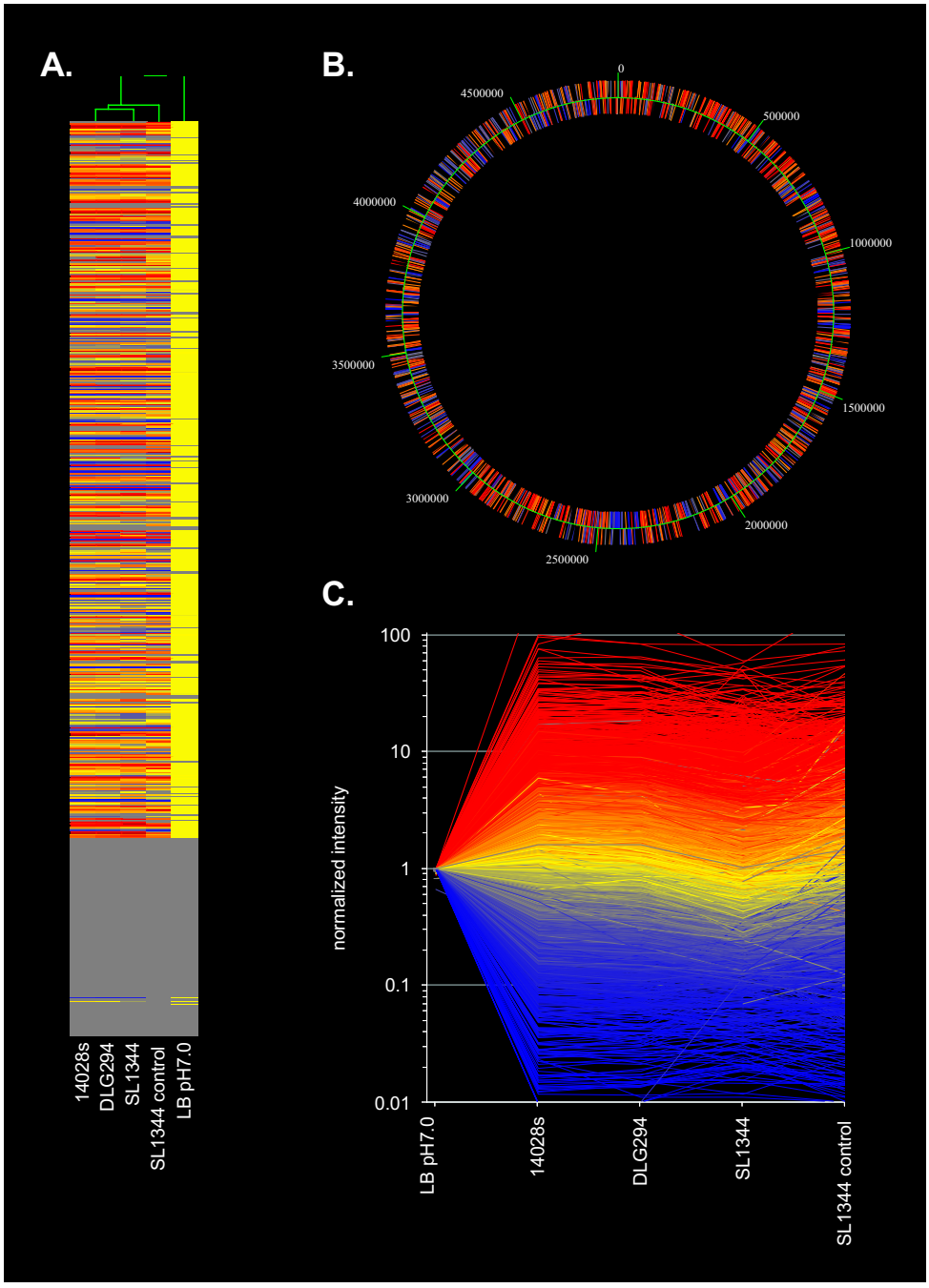

Figure 3. Cluster diagram (A), gene map (B), and gene expression profile (C) of the two-fold differentially expressed genes in intracellular 14028s, DLG294, SL1344 and SL1344 control compared to mid-logphase SL1344 grown in LB pH7.0. Each line represents one gene. Red indicates at least a twofold increase, yellow indicates no change, and blue indicates a minimum twofold decrease in expression.

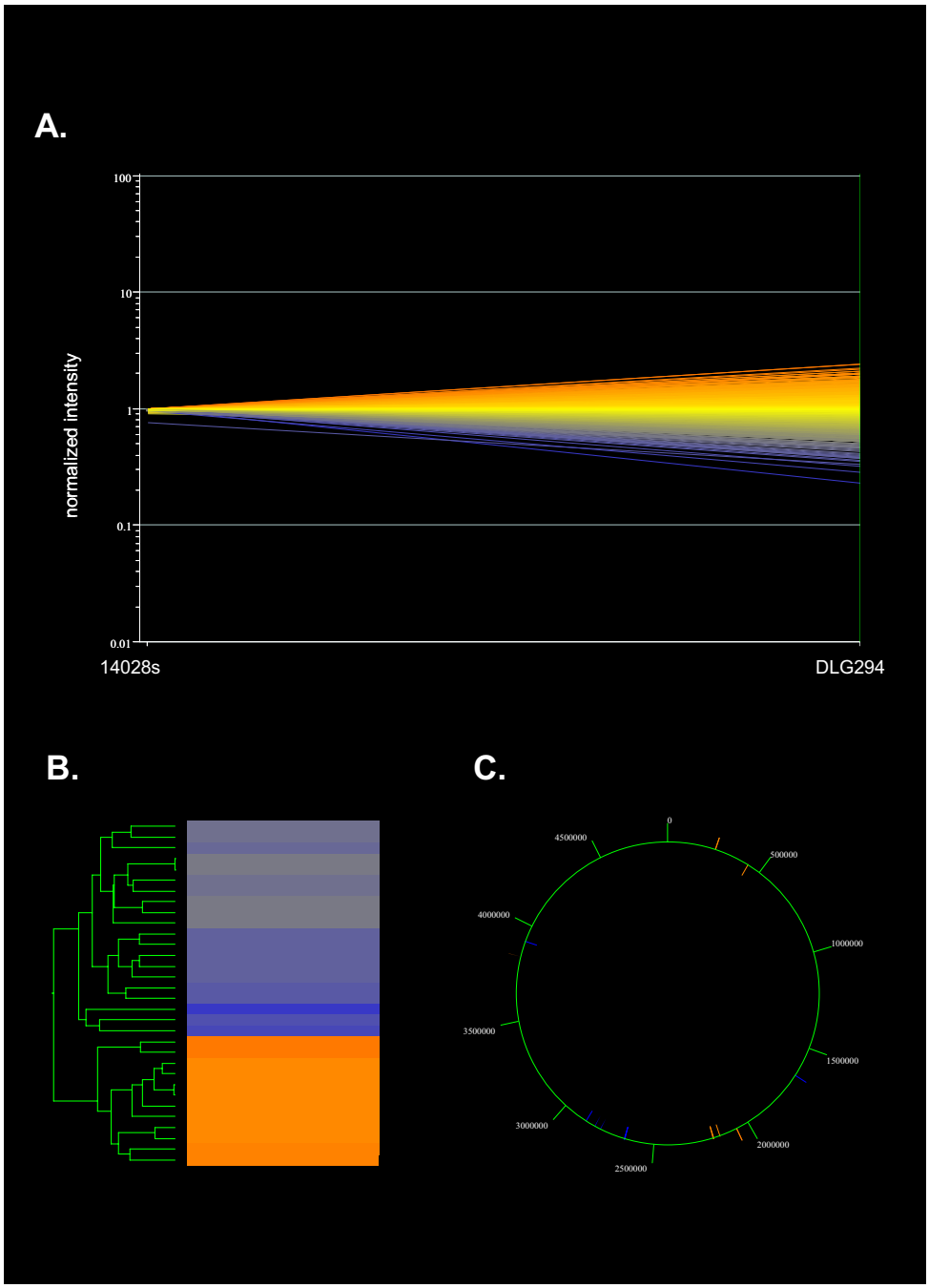

Figure 4. Relative gene expression profile (A), cluster diagram (B), and gene map (C) of two-fold differentially expressed genes in intracellular DLG294 compared to intracellular wild-type 14028s. Each line represents one gene. Red indicates at least a twofold increase, yellow indicates no change, and blue indicates a minimum twofold decrease in expression.

Genes downregulated in intracellular DLG294. The MudJ-inactivated gene *sspJ* was among the 19 down-regulated genes as well as the two genes that are located directly downstream of *sspJ,* i.e. *engA* and *yfgJ* (Fig. 5 and Table 4). These three genes, together with *yfgM*, are part of the *yfg-eng* locus in which the ORFs are all transcribed in the same direction (2, 19). The MudJ transposon did have a polar effect on the expression of *engA* and *yfgJ* since the expression of these genes was decreased in intracellular DLG294 compared to the wild-type. *EngA* encodes a GTP binding protein of which the physiolocical role is unknown and *yfgJ* encodes a putative cytoplasmic protein. The attenuated in vivo and in vitro phenotype of DLG294, however, cannot be explained by the polar effect of the MudJ transposon on these genes since complementation with a low-copy-number plasmid expressing only the *sspJ* gene completely restored the in vitro and in vivo phenotype to that of the wild-type strain and confirmed that the superoxide hypersusceptibility and attenuated phenotypes are due to the lack of expression of *sspJ* (27, 32). Recently, Amy et al. have described the attenuated in vivo phenotype of an *yfgL* (=*sspJ*) *S. enterica* serovar Enteritidis in chickens and suggested a role for the *yfg-eng* locus in colonisation of chickens (2). As for our *sspJ* mutant DLG294, this mutant showed lower bacterial numbers within macrophages after 3.5 h. The *S. enterica* serovar Enteritidis *yfgL* mutant, however, was shown to lack motility due to lack of production of the *fliC* and *fliD* encoded proteins flagellin and filament capping protein as well as the SPI-1 encoded protein SipA. For our mutant DLG294, however, no decrease in gene expression of *fliC* and *fliD* was observed nor did we observe differences in SPI-1 or SPI-2 encoded genes. DLG2294 even showed an increase in expression of genes involved in flagellar biosynthesis as the expression of the genes encoding the flagellar biosynthesis proteins FliP and FliR were increased for DLG294 compared to the wild-type strain (Fig. 5 and Table 4) and could suggest enhanced production of these inner membrane proteins involved in flagellar biosynthesis (19).

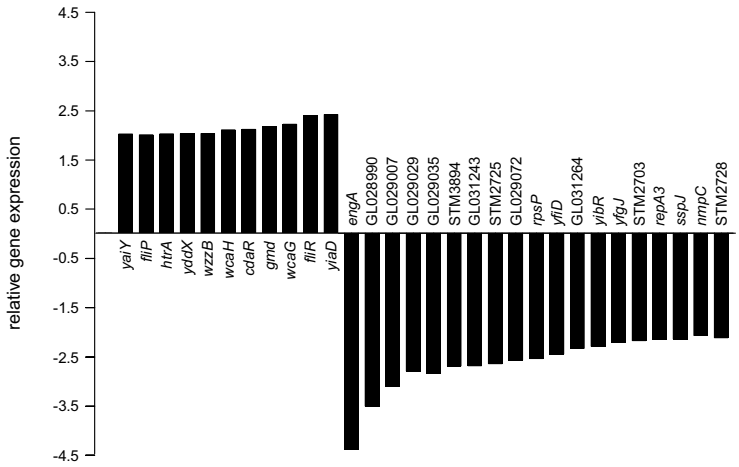

Figure 5. Fold change in gene expression of selected genes that were 2-fold differentially expressed in intracellular DLG294 compared to intracellular wild-type 14028s.

Other genes of which the expression was down-regulated in DLG294 are *rpsP* encoding 30S ribosomal subunit protein 16S, *yfiD* encoding a putative formate acetyltransferase, *yibR* encoding a putative inner membrane protein, *nmpC* encoding a predicted bacterial porin, and several other of which the gene name and function are unknown or that are orthologues to certain *E. coli* genes (Table 4). For most of these genes it is hard to predict their role in attenuated virulence of DLG294. *RepA3*, however, is a gene that is lower in DLG294 compared to the wild-type strain and that could have influenced the attenuation of DLG294 since this gene is present on the pSLT plasmid. This plasmid pSLT is the virulence plasmid of *S. enterica* strains (1) that enhances the growth rate of the bacterium during systemic phase of disease (11). The gene *repA3* is encoding a protein that is involved in the initiation of replication of this plasmid. Reduced plasmid replication might lead to reduction of virulence of the mutant strain. However, the expression of genes present on the plasmid are not altered compared to wild-type and are all upregulated to a similar extent as for the SL1344 strain in J774A.1 cells (7).

Table 4. Up- and downregulated genes in intracellular DLG294 vs 14028s

Downregulated		Upregulated	
gene	function	gene	function
enA	putative GTP-binding protein	*yaiY*	putative inner membrane protein
GL028990	no orthologues	*fliP*	flagellar biosynthesis
GL029007	orthologous to *pepA*	*htrA*	periplasmic serine protease
GL029029	orthologous to *E. coli yjhP*	*yddX*	putative cytoplasmic protein
GL029035	no orthologues	*wzzB*	putative regulator of length of O-antigen component of LPS chains
STM3894	unknown	*wcaH*	GDP-mannose mannosyl hydrolase in colanic acid biosynthesis
GL031243	putative flutathione S-transferase	*cdaR*	putative inner membrane protein
STM2725	unknown	*gmd*	GDP-D-mannose dehydratase in colanic acid biosynthesis
GL029072	putative PTS permease	*wcaG*	bifunctional GDP fucose synthetase
rpsP	30S ribosomal subunit S16	*fliR*	putative flagellar biosynthetic protein
yfiD	putative formate acetyltransferase	*yiaD*	putative outer membrane lipoprotein
GL031264	orthologous to *E. coli yeiG*		
yibR	outative inner membrane protein		
yfgJ	putative cytoplasmic protein		
STM2703	Fels2 prophage; similar to invertase (pin) in phage E14		
repA3	DNA replication		
yfgL/sspJ	putative serine/threonine kinase		
nmpC	predicted bacterial porin (outer membrane protein)		
STM2728	Fels-2 prophage; hypothetical protein		

Genes upregulated in intracellular DLG294. Only 11 genes showed increased expression in intracellular DLG294 (Fig 5 and Table 4). Two genes (*fliP* and *fliR*) are involved in flagellar biosynthesis as mentioned above. Again, for four genes, i.e. *yaiY*, *yddX*, *cdaR*, and *yiaD* encoding putative inner membrane, outer membrane, and cytoplasmic proteins (Table 4), the exact function of the proteins is unknown. Three of the

induced genes (*wcaH*, *gmd*, and *wcaG*) are involved in colanic acid biosynthesis. *WcaH* encodes the GDP-mannose mannosyl transferase, *gmd* the GDP-D-mannose dehydratase, and *wcaG* the bifunctional GDP fucose synthetase. Colanic acid, or M(ucous) antigen, is a repeat unit polysaccharide that forms a capsule and that is produced by most enteric bacteria, presumably as a means of protection against desiccation. The gene expression profiles from 14028s and DLG294 in comparison with the reference showed that the expression of these three genes was induced in intracellular bacteria and that this was higher for the superoxide-hypersusceptible mutant DLG294. This could suggest that the mutant produces more colanic acid for protection against the influx of damaging agents that act inside the macrophage as a kind of compensatory mechanism.

The gene *htrA*, encoding a periplasmic serine protease, is induced in intracellular DLG294 compared to wild-type. It has been described previously that inactivation of *htrA* or inactivation of *rpoE,* the sigma factor identified in *E. coli* as being important for survival under extreme heat-stress and that controls *htrA*, leads to a phenotype that is identical to that of DLG294 (26). These mutants display increased susceptibility to superoxide and are attenuated in mice and that, just like the *sspJ* mutant DLG294, are able to cause fatal infections in mice deficient in NADPH oxidase as well (21).(15) One could explain sensitivity of the mutants to exogenous superoxide by assuming that exposure to superoxide results in direct damage to periplasmic proteins. Elimination of these damaged proteins by HtrA, the periplasmic protease which is under control of *rpoE*, would then protect against this damage. The increase in *htrA* expression in DLG294 could suggest that this mutant imagines that it is under extreme heat stress and starts producing this heat stress protein since the conditions inside the bacteria are not right as was suggested by the lost phenotypes in the in vitro phenotype array (Table 2). Increased expression of *htrA* could also suggest a mechanism to try and compensate for the lack of expression of *sspJ*, although this "compensatory" mechanism is not sufficient to protect against oxidative damage and to restore virulence since DLG294 is still hypersusceptible to superoxide and displays an attenuated in vitro and in vivo phenotype.

Concluding remarks. DLG294 has a completely different phenotype compared to the wild-type. It is hypersusceptible to intracellular superoxide generated by menadione. It is attenuated in macrophages and in mice and regains virulence in $p47^{phox-/-}$ mice. However, when we compared gene expression profiles of intracellular DLG294 and wild-type 14028s we found that the expression profiles were only slightly altered. This indicates that *sspJ* does not have an important role in regulating (virulence) gene expression but that it has a direct role in survival under stress conditions like those encountered inside the macrophage. We propose that the absence of SspJ alters the integrity of the *Salmonella* membrane leading to increased permeability and subsequent accumulation of toxic compounds. This in turn would lead to enhanced susceptibility to these toxic compounds (like menadione) and antibiotics and to the attenuated in vivo phenotype of DLG294. The small differences in gene expression probably reflect a difference in intracellular environment as a result of this increased membrane permeability.

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