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Inactivation of alternative sigma factor 54 (RpoN) leads to increased acid resistance, and alters locus of enterocyte effacement (LEE) expression in *Escherichia coli* O157 : H7

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Alternative sigma factor 54 (RpoN) is an important regulator of stress resistance and virulence genes in many bacterial species. In this study, we report on the gene expression alterations that follow *rpoN* inactivation in *Escherichia coli* O157 : H7 strain Sakai (Sakai*rpoN* : : *kan*), and the influence of RpoN on the acid resistance phenotype. Microarray gene expression profiling revealed the differential expression of 103 genes in Sakai*rpoN* : : *kan* relative to Sakai. This included the growth-phase-dependent upregulation of genes required for glutamate-dependent acid resistance (GDAR) (*gadA*, *gadB*, *gadC* and *gadE*), and the downregulation of locus of enterocyte effacement (LEE) genes, which encode a type III secretion system. Upregulation of *gad* genes in Sakai*rpoN* : : *kan* during exponential growth correlated with increased GDAR and survival in a model stomach system. Complementation of Sakai*rpoN* : : *kan* with a cloned version of *rpoN* restored acid susceptibility. Genes involved in GDAR regulation, including *rpoS* (sigma factor 38) and *gadE* (acid-responsive regulator), were shown to be required for the survival of Sakai*rpoN* : : *kan* by the GDAR mechanism. This study describes the contribution of *rpoN* to acid resistance and GDAR gene regulation, and reveals RpoN to be an important regulator of stress resistance and virulence genes in *E. coli* O157 : H7.

INTRODUCTION

Escherichia coli O157 : H7 is an important aetiological agent of food-borne diarrhoea, bloody diarrhoea and haemolytic uraemic syndrome (Mead & Griffin, 1998; Mead *et al.*, 1999). Infection with *E. coli* O157 : H7 is characterized by a dramatic intestinal histopathology termed attaching and effacing (A/E) lesions. These lesions result from the intimate adherence of *E. coli* O157 : H7 to host intestinal cells, forming cup-like pedestals on which the bacteria are intimately perched (Donnenberg & Whittam, 2001). The determinants of the A/E phenotype are encoded on a 35.6 kb laterally acquired pathogenicity island termed the locus of enterocyte effacement (LEE) (Perna *et al.*, 1998). The LEE encodes a type III secretion apparatus, which is required for the translocation of virulence factors (effectors) from *E. coli* O157 : H7 into host enterocytes (Perna *et al.*, 1998).

For successful transmission, *E. coli* O157 : H7 is dependent on the expression of acid resistance, which allows for passage through the acid barrier of the stomach and low oral infectious dose (Chart, 2000; Teunis *et al.*, 2004). The *E. coli* acid resistance phenotype is characterized by protracted survival under conditions of extreme acid stress. More specifically, cells are considered acid resistant if survival following 2 h of acid challenge (pH 2.0–2.5) exceeds 10 % of initial cell densities (Gorden & Small, 1993). These conditions are representative of the retention time of food in the stomach, and the pH of stomach gastric

Abbreviations: A/E, attaching and effacing; GDAR, glutamate-dependent acid resistance; GSEA, gene set enrichment analysis; LEE, locus of enterocyte effacement; SAM, significance analysis of microarrays.

Normalized microarray intensity data files have been deposited in the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) with accession number GSE17467.

Two supplementary tables and a supplementary figure are available with the online version of this paper.

acid. There are three major systems of acid resistance in *E. coli* O157 : H7: arginine- and glutamate-dependent systems, and an oxidative system (Foster, 2004). Of these three, the glutamate-dependent acid resistance (GDAR) system is believed to provide the highest protection from acid stress. GDAR requires exogenous glutamate, glutamate decarboxylase (GadA and GadB isoforms), and an antiporter (GadC), which exchanges the decarboxylation product, γ-aminobutyrate, for fresh glutamate (Castanie-Cornet *et al.*, 1999). Regulation of the GDAR system is multifactorial, and partly includes alternative sigma factor 38 (RpoS), the nucleoid structuring protein H-NS, the AraC/XylS-like transcriptional regulators GadX and GadW, and the LuxR-like regulator GadE (De Biase *et al.*, 1999; Ma *et al.*, 2002, 2003; Masuda & Church, 2003; Tramonti *et al.*, 2002).

Alternative sigma factor 54 (RpoN) is one of seven RNA polymerase sigma subunits in *E. coli* required for promoter-initiated transcription. RpoN plays a major role in the response of *E. coli* to nitrogen-limiting conditions. Under such conditions, RpoN directs the transcription of at least 14 *E. coli* operons/regulators in the nitrogen regulatory (Ntr) response (Reitzer & Schneider, 2001). RpoN also plays a role in various stress response mechanisms of *E. coli*. For instance, the *E. coli* phage-shock operon (*psp*) is driven by an RpoN-dependent promoter and protects *E. coli* from alkaline pH during stationary-phase growth (Model *et al.*, 1997). In addition, transposon insertion in *rpoN* has been shown to result in elevated resistance to the DNA gyrase inhibitor novobiocin in *E. coli* (Jovanovic *et al.*, 1999). RpoN has also been shown to be important for stress resistance in bacteria other than *E. coli*. For example, inactivation of *rpoN* in *Listeria monocytogenes* affects its ability to grow under osmotic stress (Okada *et al.*, 2006), and leads to increased resistance to certain bacteriocins in *L. monocytogenes* and *Enterococcus faecalis* (Dalet *et al.*, 2000; Robichon *et al.*, 1997). In addition, RpoN has been determined to regulate virulence gene expression. This includes type III secretion in *Pseudomonas aeruginosa*, and O-antigen expression in *Salmonella enterica* (Bittner *et al.*, 2002; Matz *et al.*, 2008). Lastly, RpoN has been reported to co-regulate various bacterial virulence genes in conjunction with RpoS (Bittner *et al.*, 2004; Yang *et al.*, 2005).

This study investigated the RpoN regulon in the 1996 Osaka Japan outbreak *E. coli* O157 : H7 strain Sakai (Michino *et al.*, 1999), using gene expression microarray analysis. Our results revealed RpoN to be a new regulator of the LEE, and of acid-resistance genes of the GDAR system. The effect of RpoN on the phenotypic expression of acid resistance, the regulation of GDAR genes, and the role for GDAR system regulators in RpoN-dependent repression of acid resistance was also examined.

METHODS

Bacterial strains, genetic manipulations, and growth conditions. The strains and plasmids used in this study are listed in Table 1. Isogenic and polygenic mutants were constructed using the λ-Red recombinase-assisted one-step inactivation method adapted for enterohaemorrhagic *E. coli* (Murphy & Campellone, 2003). Briefly, primers P1 and P2, which amplify a kanamycin (Kan) resistance cassette from plasmid pKD4 (Datsenko & Wanner, 2000), were constructed with 40 bp oligonucleotide 5′ extensions, which were homologous to the up- and downstream intergenic regions of genes targeted for inactivation (see Supplementary Table S1, available with the online version of this paper, for details of all primers). These primers were used to generate a PCR product, which was then electroporated (2.5 kV, 5.6 ms) using a MicroPulser electroporator (Bio-Rad) into a Red-recombinase-producing background of *E. coli* O157 : H7 strain Sakai, as described previously (Kailasan Vanaja *et al.*,

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Source/reference
***E. coli* O157 : H7**		
TW08264	WT strain Sakai	Michino *et al.* (1999)
EcJR-8	Sakai*rpoN*::*kan*	This study
EcJR-5	Sakai*rpoN*::*kan* pCR2.1(*rpoN*$^+$)	This study
EcJR-6	SakaiΔ*gadE rpoN*::*kan*	This study
EcJR-9	SakaiΔ*rpoS rpoN*::*kan*	This study
TW04863	WT strain 93-111	STEC Center, Mich. State Univ.
EcJR-10	93-111*rpoN*::*kan*	This study
TW14359	WT	STEC Center, Mich. State Univ.
EcJR-11	TW14359*rpoN*::*kan*	This study
TW15901	Sakai pKM208	Kailasan Vanaja *et al.* (2009)
TW15902	SakaiΔ*gadE*	Kailasan Vanaja *et al.* (2009)
Plasmids		
pCR2.1	TOPO cloning vector	Invitrogen
pCR2.1(*rpoN*+)	pCR2.1 containing *rpoN*	This study
pKM208	Red-recombinase expression vector	Datsenko & Wanner (2000)
pKD4	Template plasmid for Kan cassette	Datsenko & Wanner (2000)
pCP20	Flp recombinase expression vector	Datsenko & Wanner (2000)

2009). Transformants were selected on Luria–Bertani (LB) 1.5 % (w/v) agar plates with Kan (20 μg ml^{-1}). Removal of Kan cassettes was performed by introducing pCP20, encoding a Flp recombinase, into isogenic backgrounds by electroporation following the method of Datsenko & Wanner (2000). Polygenic mutants were constructed by inactivating *rpoN* in Δ*rpoS* and Δ*gadE* isogenic backgrounds to produce SakaiΔ*rpoS rpoN*::*kan* and SakaiΔ*gadE rpoN*::*kan*. Sakai*rpoN*::*kan* was complemented *in trans* with the wild-type Sakai *rpoN* ORF. This was generated by PCR with TaKaRa LA *Taq* polymerase, and cloned into pCR2.1 (Invitrogen) to produce Sakai*rpoN*::*kan* pCR2.1(*rpoN*$^{+}$). All genetic constructs were confirmed by PCR and restriction mapping. In addition, impaired growth and glutamine auxotrophy for *rpoN* mutants (Reitzer *et al.*, 1987) were confirmed in DMEM-MOPS (Supplementary Fig. S1), and by the absence of growth in minimal medium without glutamine, respectively. Diminished catalase activity and glycogen storage of *rpoS* mutants was confirmed for SakaiΔ*rpoS rpoN*::*kan* according to previously designed methods (Bohannon *et al.*, 1991; Hengge-Aronis & Fischer, 1992).

For all experiments, LB starter cultures were inoculated with single colonies and grown to an OD_{600} of 0.5 at 37 °C (180 r.p.m.). These cultures were then used to inoculate (0.5 %, v/v, inoculum) 50 mM MOPS-buffered Dulbecco's Modified Eagle's Medium containing 4 g glucose l^{-1} (pH 7.4) (DMEM-MOPS), LB containing 50 mM MOPS (pH 7.4), LB containing 50 mM MOPS and 4 g glucose l^{-1} (LBG-MOPS) (pH 7.4), or LB with 4 g glucose l^{-1} (LBG) (pH 7.4), and grown overnight at 37 °C (180 r.p.m.). DMEM is a rich but defined medium, which contains glutamine to support the growth of *rpoN* mutants, which are auxotrophic for glutamine. These overnight cultures were then used to inoculate various growth media (initial inoculum $OD_{600} \approx 0.05$) for all experiments. The growth of DMEM-MOPS cultures utilized for RNA isolation, DNA microarray analysis and quantitative real-time PCR (qRT-PCR) experiments were monitored by taking OD_{600} readings at 1 h intervals for 8 h (Supplementary Fig. S1).

DNA microarrays. Microarray analysis was performed to determine transcriptome alterations that occur in *E. coli* O157 : H7 Sakai as a result of *rpoN* inactivation in exponential- and early stationary-phase cultures. DNA microarrays consisted of 6088 spotted 70-mer oligonucleotides (*E. coli* Oligo set version 1.0.1, Qiagen) representing ORFs from *E. coli* O157 : H7 strains EDL-933 and Sakai, and K-12 strain MG1655. Over 1700 ORFs were specific to *E. coli* O157 : H7, and included 85 ORFs from pO157 and three ORFs from pOSAK1. DMEM-MOPS cultures (n=4) were sampled during exponential (OD_{600} 0.5) and early stationary-phase growth (OD_{600} 1.8), and RNA was extracted as described previously (Bergholz *et al.*, 2007b). Amino-allyl labelling and cDNA synthesis, cy-dye coupling and hybridization conditions followed previously described methods (Bergholz *et al.*, 2007b). Image files (TIFF) of 16 hybridized microarray slides (four slides per strain, per phase of growth) were generated using an Axon 4000B scanner (Molecular Devices), and analysed using GenePix Pro software (Molecular Devices, version 6.0). The resulting microarray intensity data were $\log_2$-transformed, and normalized using the LOWESS algorithm in MAANOVA version 0.98-8 (R version 2.2.1). Statistical analysis was performed using a significance analysis of microarrays (SAM, MultiExperiment Viewer, version 4.0) (Tusher *et al.*, 2001) unpaired contrast, available through the TM4 software package (JCVI). A *d*-statistic (Tusher *et al.*, 2001) was calculated for each gene based on repeated permutations, and a false discovery rate (FDR) of 0.05 was used to assign a critical cutoff for significance. Gene expression ratios (R) were calculated for each significant gene using $R = 2^{\log_2(\text{Sakai}ropN::kan) - \log_2(\text{Sakai})}$, and were considered biologically significant using a cutoff of $0.5 \geqslant R \geqslant 2.0$. Gene set enrichment analysis (GSEA) (Subramanian *et al.*, 2005) was also used on select microarray datasets of gene clusters that shared similar biological function/regulation. Normalized microarray intensity data files have been deposited in the Gene Expression Omnibus (GSE17467) through NCBI.

qRT-PCR. For microarray validation, DMEM-MOPS cultures (n=3) were sampled for RNA extraction, as described above. Sampling was also performed as described above at OD_{600} 0.2, 0.4, 0.6, 0.8 and 1.2, to examine the expression of GDAR genes in Sakai and Sakai*rpoN*::*kan* during exponential into transition-phase growth. cDNA was prepared from 1 μg RNA samples using iScript Select cDNA synthesis (Bio-Rad) and qRT-PCR was performed using the iQ5 system (Bio-Rad) with the primers and probes listed in Supplementary Table S1. For *gadA* and *gadB*, TaqMan probes were developed and optimized according to established protocols (Bergholz *et al.*, 2007a) and for all remaining qRT-PCR targets, SYBR chemistries and reaction conditions were used as previously described (Bergholz *et al.*, 2007b). Cycle threshold (C_t) data were normalized to *rrsA* and normalized cycle threshold values (ΔC_t) were transformed using $2^{-\Delta C_t}/10^{-6}$ (Livak & Schmittgen, 2001), and reported as arbitrary gene expression units (EU) or as the gene expression ratio of Sakai*rpoN*::*kan*/Sakai. In keeping with microarray analysis, a twofold cutoff was used for gene expression ratios to denote biologically significant changes in expression. Differences in mean EU between Sakai and Sakai*rpoN*::*kan* for individually analysed genes were compared using Welch's *t*-test (R).

Assessment of GDAR. GDAR was determined as previously described (Large *et al.*, 2005) with slight modification. DMEM-MOPS, LB-MOPS, LBG-MOPS or LBG cultures were initially grown to the OD_{600} described below, before using these cultures ($\approx$50 μl culture volume) to inoculate 10 ml (10 : 1 flask to medium ratio) minimal EG medium (73 mM K_2HPO_4, 10 mM sodium citrate, 0.8 mM $MgSO_4$, 5.7 mM glutamate and 4 g glucose l^{-1}, pH 2.0) to reach a final density of 10^6 c.f.u. ml^{-1}. To determine the effects of glutamate on the survival of Sakai*rpoN*::*kan* by the GDAR mechanism, EG medium was also prepared without glutamate addition. GDAR was tested with Sakai and Sakai*rpoN*::*kan* cultures grown to OD_{600} 0.5, 0.75, 1.0 and 3.5; all remaining strains were tested at OD_{600} 0.5 only. Samples were drawn from EG cultures (180 r.p.m., 37 °C) at 0.5 h intervals and diluted before adding aliquots of the dilutions to LB agar, followed by incubation at 37 °C for 18–20 h before determining c.f.u. ml^{-1}. Survival rates (ΔV) were extrapolated from plate count data using linear regression analysis (R), and were reported as the $\log_{10}$ c.f.u. decrease per h (log c.f.u. h^{-1}). The strength of linearity was estimated by the correlation coefficient (r^2), which exceeded 0.85 (85 %) for all analyses. Differences in mean ΔV between wild-type and mutant derivative strains were examined statistically using Welch's *t*-test.

Assessment of acid resistance in a model stomach system. Acid resistance in a model stomach system was determined as previously described (Bergholz & Whittam, 2007), with the following adaptations. DMEM-MOPS cultures were grown to OD_{600} 0.5 before using these cultures to inoculate 10^6 c.f.u. ml^{-1} into simulated gastric fluid (pH 2) mixed with 30 g Gerber Turkey baby food using a Stomacher 400C (Seward). Aliquots were recovered from this system at 1 h intervals for a total of 2 h for plate counts on LB agar. Mean counts for Sakai and Sakai*rpoN*::*kan* were then compared using Welch's *t*-test.

RESULTS

Gene expression profile of *rpoN*-inactivated *E. coli* O157 : H7 strain Sakai

We used microarray analysis to examine the impact of *rpoN* inactivation in *E. coli* O157 : H7 Sakai

Table 2. Differentially expressed genes in Sakai*rpoN*::*kan* compared to Sakai

ECs no.*	Gene	Function	Expression ratio Sakai*rpoN*::*kan*/Sakai†	
			Expo	Stat
Hypothetical and miscellaneous				
0069	*yabI*	Membrane-associated protein	0.20	–
0781	*ybgS*	Homeobox protein	7.73	–
0982	*ycaC*	Predicted hydrolase	9.24	–
1152	*yccJ*	Unknown	3.24	–
1683	*ycgB*	Putative sporulation protein	5.59	–
1695	*yihV*	Unknown	–	0.05
1768‡		Sp9-encoded protein	4.21	–
2316	*tus*	DNA-replication protein	3.63	–
2430	*ydiZ*	Unknown	3.88	–
2546	*yebV*	Unknown	20.0	–
2547	*yebW*	Unknown	5.85	–
2662	*fliC*	Flagellin	0.25	–
2692	*yodD*	Unknown	6.94	–
3154	*elaB*	Unknown	12.1	–
3413‡		Unknown	5.32	–
4037	*yhbQ*	Predicted endonucleases	4.44	–
4291‡		Unknown	8.59	–
4323	*yhhT*	Inner-membrane protein	0.07	0.30
4363	*yhiM*	Unknown	6.48	–
4699	*yifE*	Unknown	0.12	–
4737	*yzcX*	Unknown	5.81	–
4745	*yigE*	Unknown	2.65	–
4801‡		Unknown	3.17	–
Stress resistance				
1829	*yciE*	Unknown	6.00	–
1830	*yciF*	Structural protein	13.5	–
1831	*yciG*	Unknown	9.39	–
1881§	*pspA*	Phage-shock protein A	0.28	0.04
1882§	*pspB*	Phage-shock protein B	–	0.14
1883§	*pspC*	Phage-shock protein C	–	0.34
1884§	*pspD*	Phage-shock protein D	–	0.36
1885§	*pspE*	Phage-shock protein E	–	0.49
2097	*gadC*	Acid-sensitivity protein (XasA)	5.28	–
2098	*gadB*	Glutamate decarboxylase isozyme	4.07	–
2604	*otsA*	Trehalose-6-phosphate synthase	4.46	–
2605	*otsB*	Trehalose-6-phosphate phosphatase	2.82	–
3186	*yfcG*	Glutathione *S*-transferase	3.23	–
3533	*ygaM*	Unknown	6.62	–
4377	*slp*	Starvation lipoprotein	3.29	–
4392	*gadE*	Acid-responsive regulator	3.83	–
4396	*gadX*	Glutamate decarboxylase activator	3.31	–
4397	*gadA*	Glutamate decarboxylase isozyme	3.49	–
5586	*ecnB*	Bacteriolytic enterocidin B	7.47	–
Transport and metabolism				
0504§	*glnK*	Nitrogen regulatory protein P-II 2	0.01	0.02
0505§	*amtB*	High-affinity ammonium transporter	0.08	0.12
0538	*ybaS*	Glutaminase	5.03	–
0693§	*gltJ*	Glutamate transporter, permease	0.19	–
0694§	*gltI*	Glutamate transporter, periplasmic	0.08	0.07
0887§	*glnQ*	ATP-binding protein for Gln transporter	0.26	–
0888§	*glnP*	Permease protein of Gln transporter	–	0.30
0889§	*glnH*	Periplasmic protein for Gln transporter	0.07	0.09

Table 2. cont.

ECs no.*	Gene	Function	Expression ratio Sakai*rpoN*::*kan*/Sakai†	
			Expo	Stat
1254§	*rutE*	Nitroreductase	0.19	–
1255§	*rutD*	Putative acetyltransferase	0.24	–
1711	*ychM*	Sulfate permease	5.73	–
1722	*chaB*	Cation transport regulator	7.70	–
1879	*puuE*	GABA-aminotransferase	4.06	–
1921	*abgB*	*p*-Aminobenzoyl-glutamate hydrolase	–	0.22
1922	*abgA*	Putative aminohydrolase	–	0.06
2082	*adhP*	Ethanol dehydrogenase	7.50	–
2091§	*ddpA*	Putative D-Ala-D-Ala transport protein	0.09	–
2092§	*ddpX*	Putative D-Ala-D-Ala dipeptidase	0.09	–
2103	*ydeN*	Predicted sulfatase	0.12	–
2451§	*astB*	Succinylarginine dihydrolase	0.25	–
2452§	*astD*	Succinylglutamic semialdehyde dehydrolase	0.15	–
2453§	*astA*	Arginine succinyltransferase	0.21	–
2454§	*astC*		0.20	–
2650	*pgsA*	Phosphatidylglycerophosphate synthetase	2.93	–
2784§	*nac*	Nitrogen assimilation control protein	0.03	0.08
2900		Fructose bisphosphate aldolase	7.65	–
3058	*yeiC*	Predicted kinase		0.27
3192§	*hisQ*	Permease for histidine transport	0.28	–
3193§	*hisJ*	Periplasmic protein for histidine transport	0.15	–
3327	*tktB*	Transketolase	8.50	–
3425	*tadA*	tRNA-specific adenosine deaminase	5.13	–
3689	*ygdQ*	TerC-like transport protein	0.06	0.09
4141§	*yhdW*	Periplasmic-binding protein	0.38	–
4142§	*yhdX*	Transport system permease protein	0.46	–
4144§	*yhdZ*	ATP-binding protein	0.29	–
4448	*xylA*	Xylose isomerase	0.17	–
4490	*yibO*	Phosphoglyceromutase	7.18	–
4492	*yibQ*		2.48	–
4734	*hemD*	Uroporphyrinogen-III synthetase	2.54	–
4735	*hemC*	Porphobilinogen deaminase	2.07	–
4790§	*glnG*	Glutamine utilization response regulator	0.15	–
4791§	*glnL*	Glutamine utilization sensor kinase	0.08	0.34
4792§	*glnA*	Glutamine synthetase	0.08	0.07
Transcription and translation				
1902	*tyrR*	Transcriptional dual regulator	6.36	–
2032	*rimL*	Ribosomal-serine *N*-acetyltransferase	4.59	–
2084	*rpsV*	30S ribosomal protein S22	6.34	–
2783	*cbl*	CysB-like regulator of *cys* operon	0.09	0.18
3136	*yfaX*	Putative regulator	0.12	–
3403	*hcaR*	Transcriptional activator of *hca* cluster	2.75	–
4440	*yiaG*	Transcriptional regulator	7.66	–
4484	*yibK*	rRNA methylase	4.51	–
4867	*metJ*	Methionine transcriptional repressor	2.40	–
Pathogenesis				
0780	*zitB*	Zinc transport/adhesin	0.36	–
3704‡	*yqeI*	ETT2 sensory transducer	3.91	–
4551‡	*orf29*	*LEE4* type III secretion protein	0.33	–
4554‡	*espB*	*LEE4* translocon protein	0.29	–
4560‡	*cesT*	*LEE5* type III secretion chaperone	0.17	–
4561‡	*tir*	*LEE5* translocated intimin receptor	0.26	–
4563‡	*cesF*	*LEE3* type III secretion chaperone	0.32	–

Table 2. cont.

ECs no.*	Gene	Function	Expression ratio Sakai*rpoN*::*kan*/Sakai†	
			Expo	Stat
4491	*envC*	Murien hydrolase	4.31	–
4571‡	*espZ*	*LEE2* type III secretion protein	0.24	–

*Locus tag (ECs no.) for *E. coli* O157 : H7 strain Sakai (GenBank no. BA000007).

†Expression ratio for exponential (Expo) and early stationary (Stat) phase cultures of Sakai*rpoN*::*kan* and Sakai determined as $2^{\log_2(\text{Sakai}rpoN::kan) - \log_2(\text{Sakai})}$. –, No significant differential expression between Sakai*rpoN*::*kan* and Sakai.

‡Gene is encoded within an *E. coli* O157 : H7-specific sequence.

§Gene previously shown to be regulated by RpoN.

(Sakai*rpoN*::*kan*) on global gene expression during exponential- and early stationary-phase growth in DMEM-MOPS. Inactivation of *rpoN* resulted in the differential expression of 103 ORFs (Table 2). This altered expression was growth phase dependent, with 95 ORFs being differentially expressed in exponential phase, and 19 in early stationary phase. Fifty-four exponential-phase ORFs were upregulated in Sakai*rpoN*::*kan*, whereas all 19 ORFs altered in early stationary phase were downregulated. Only 11 ORFs were found to be differentially expressed in both growth phases. Overall, our microarray analysis identified 74 ORFs that have not been shown previously to be regulated by RpoN in *E. coli* (Table 2). The majority of differentially expressed ORFs (43/103) represented genes involved in nitrogen assimilation, as well as genes involved in the metabolism and transport of carbohydrates and inorganic compounds. As *rpoN* is required for stimulation of the nitrogen regulatory response in nitrogen-limiting media, such as DMEM (Reitzer & Schneider, 2001), genes involved in nitrogen uptake and assimilation were downregulated in Sakai*rpoN*::*kan* relative to Sakai. These included *glnALG*, *glnHPQ*, *glnK-amtB*, *gltIJKL*, *yhdWXYZ*, *astCADBE* and *nac* (Table 2). These operons and genes are involved in glutamine biosynthesis, glutamine transport, ammonia transport, glutamate transport, ABC-type transport, arginine metabolism and nitrogen assimilation, respectively.

RpoN regulates the expression of stress resistance genes

Nineteen ORFs determined to be differentially expressed in Sakai*rpoN*::*kan* by microarray analysis encoded gene products associated with stress resistance (Table 2). Interestingly, 14/19 stress-resistance genes were upregulated in exponential-phase Sakai*rpoN*::*kan* cultures. These included numerous genes shown to be induced upon exposure to osmotic stress, such as bacteriolytic enterocidin B (*ecnB*), which is activated under high-osmolarity conditions (Bishop *et al.*, 1998); *otsBA*, which encodes the enzymes trehalose-6-phosphate phosphatase and trehalose-6-phosphate synthase, involved in metabolism of the osmoprotectant trehalose (Giaever *et al.*, 1988; Hengge-Aronis *et al.*, 1991); and *yciGFE*, encoding predicted or conserved proteins, the function of which is unknown (Weber *et al.*, 2005) (Table 2). In addition, *yfcG* (Dong *et al.*, 2007), which encodes a glutathione *S*-transferase (Wadington *et al.*, 2009) required for hydrogen peroxide resistance (Kanai *et al.*, 2006), was upregulated in Sakai*rpoN*::*kan*. Two genes induced upon exposure to moderate acid stress, *ygaM* and *slp* (Tucker *et al.*, 2002; Weber *et al.*, 2005), were also upregulated in Sakai*rpoN*::*kan* in exponential phase. As expected, the phage-shock operon, encoded by *pspABCDE* and transcriptionally regulated by RpoN (Weiner *et al.*, 1991), was downregulated in stationary-phase Sakai*rpoN*::*kan* cultures (Table 2). *E. coli psp* mutants demonstrate poor survival in stationary-phase alkaline cultures, reduced biofilm formation, and deficiencies in proton-motive force and protein secretion (Darwin, 2005). Many of these stress-resistance-associated genes (*ecnB*, *otsBA*, *yciGFE*, *yfcG* and *ygaM*) have been previously shown to be regulated by RpoS (Bishop *et al.*, 1998; Giaever *et al.*, 1988; Hengge-Aronis *et al.*, 1991; Kanai *et al.*, 2006; Tucker *et al.*, 2002; Weber *et al.*, 2005).

RpoN positively regulates LEE gene expression

Microarray analysis using significance analysis of microarrays (SAM) identified 11 *E. coli* O157 : H7-specific ORFs to be differentially expressed in exponential-phase Sakai*rpoN*::*kan* compared to Sakai, which included six downregulated ORFs encoded on the LEE (Table 2). The five remaining *E. coli* O157 : H7-specific genes encoded an *E. coli* type III secretion system 2 sensory transducer (*yeqI*), a prophage-borne gene (ECs1768), and three genes encoding hypothetical proteins: ECs3413, ECs4291 and ECs4801. The LEE genes downregulated in exponential-phase Sakai*rpoN*::*kan* encoded a protein required for the translocation of type III secretion effectors (*espZ*) and a secreted structural component of the translocation apparatus (*espB*); the translocated intimin receptor (*tir*) and a

chaperone required for proper secretion of Tir (*cesT*); a chaperone (*cesF*) required for proper folding of EspF; and *orf29*, which has no known function (reviewed by Crawford *et al.*, 2002). The LEE is composed of 41 ORFs, and is essential to the pathogenesis of *E. coli* O157 : H7. We therefore utilized gene set enrichment analysis (GSEA) to determine if the expression of other LEE-encoded genes was also altered by *rpoN* inactivation. GSEA identified 28 genes encoded throughout the LEE to be significantly downregulated in Sakai*rpoN*::*kan*, with a normalized enrichment score (NES) of −2.8 (FDR *q* value=0.0; Fig. 1). This set included those genes determined to be differentially expressed by SAM alone. Collectively, the results of SAM and GSEA analysis of microarray data indicate that genes within all five of the LEE operons, including the LEE regulator *grlA*, were downregulated in exponential-phase cultures of Sakai*rpoN*::*kan* relative to Sakai (Table 2, Fig. 1).

Validation of microarray data using qRT-PCR

qRT-PCR was used to independently validate gene expression differences for selected GDAR and LEE genes (Table 3). The expression of GDAR genes *gadC* and *gadE* and of LEE genes *cesT*, *espA*, *espB*, *espZ*, *ler* and *tir*, during exponential growth as determined by qRT-PCR was concordant with microarray expression results. Gene expression ratios from microarray and qRT-PCR analyses were found to be tightly correlated (r^2=0.86). The *gadC* gene, however, was substantially more upregulated in Sakai*rpoN*::*kan* as determined by qRT-PCR than by microarray analysis. In addition, *espA* was determined to be significantly downregulated in Sakai*rpoN*::*kan* by qRT-PCR analysis, but not by microarray analysis (Table 3). These disparities may reflect reported differences in sensitivity between the two techniques (Canales *et al.*, 2006).

Negative regulation of *gad* genes by RpoN

Genes essential to the GDAR system, *gadA*, *gadB* and *gadC*, and genes encoding factors that regulate the GDAR system, *gadE* and *gadX*, were all upregulated in exponential-phase Sakai*rpoN*::*kan*, as determined by microarray analysis, but were not differentially expressed in early stationary phase (Table 2). Since *gad* genes were upregulated in Sakai*rpoN*::*kan*, and the GDAR system is essential for full expression of the acid-resistance phenotype of *E. coli* O157 : H7, we decided to compare the expression of these genes in Sakai*rpoN*::*kan* and Sakai at various OD_{600} values during the exponential (OD_{600} 0.2–0.8) and transition (OD_{600} >0.8) phases of growth (Fig. 2). Transcriptional profiling of *gadA*, *gadB*, *gadC* and *gadE* by qRT-PCR revealed a significant upregulation of these genes in both Sakai*rpoN*::*kan* and Sakai during exponential-phase growth (Fig. 2a–d). All of these genes, however, were significantly more upregulated in Sakai*rpoN*::*kan* compared to Sakai (P<0.01) (Fig. 2). Upon entry into transition-phase growth, the expression of these four genes in both Sakai*rpoN*::*kan* and Sakai did not differ significantly (Fig. 2).

RpoS positively influences *gad* transcription during stationary-phase growth (Ma *et al.*, 2003), and H-NS represses these genes during exponential-phase growth (Giangrossi *et al.*, 2005; Tramonti *et al.*, 2006). Since the differential expression of *gad* genes in Sakai*rpoN*::*kan* was growth phase dependent, we wanted to determine if this was the result of altered *rpoS* or *hns* expression. Analysis by qRT-PCR, however, revealed no significant differences in the expression of *rpoS* or *hns* between Sakai*rpoN*::*kan* and Sakai (Fig. 2e, f). Therefore, despite the fact that RpoS and H-NS control the expression of *gad* genes, and RpoS regulates many of the RpoN-regulated stress-resistance genes discussed above, its does not appear that altered *rpoS* or *hns* expression contributes to the differential expression of *gad* genes in Sakai*rpoN*::*kan*.

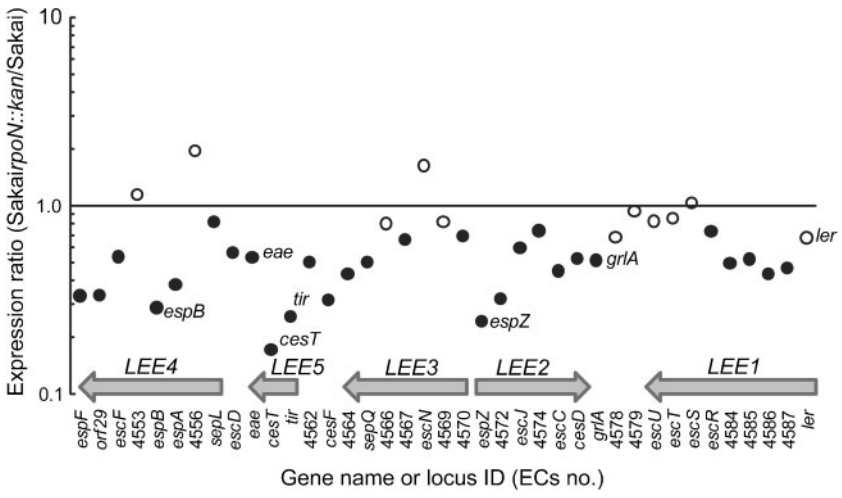

Fig. 1. Differential expression of LEE genes by GSEA analysis in Sakai*rpoN*::*kan* compared to Sakai. The fold change in gene expression is plotted against gene or locus ID (ECs no.) for all five LEE operons. Filled symbols represent genes determined to be significantly altered in expression by GSEA. All genes significant by SAM were also significant by GSEA. For orientation, names for selected LEE genes are provided to the immediate right of their respective plots.

Table 3. Validation of microarray genes by qRT-PCR

Gene	Expression ratio (Sakai*rpoN*::*kan*/Sakai)* Expo. (OD_{600}=0.5)	
	qRT-PCR	MA†
cesT	0.16±0.03‡	0.17
espA	0.29±0.06	–
espB	0.14±0.03	0.29
espZ	0.20±0.05	0.24
ler	–	–
tir	0.15±0.04	0.26
gadC	33.3±13.0	5.28
gadE	4.00±1.00	3.83

*Normalized gene expression ratios for Sakai*rpoN*::*kan*/Sakai during exponential (Expo.) phase growth in DMEM-MOPS. Gene expression ratio was calculated as $2^{\log_2(\text{Sakai}ropN::kan) - \log_2(\text{Sakai})}$. –, Not significantly altered in expression.

†MA is gene expression ratio for microarray data from Supplementary Table S2.

‡95 % confidence interval on the mean (n=3).

Effects of *rpoN* inactivation on acid resistance

Since *gad* gene expression was upregulated in Sakai*rpoN*::*kan*, we hypothesized that acid resistance would also be altered in Sakai*rpoN*::*kan*. GDAR was tested in exponential- (OD_{600} 0.5) and stationary- (OD_{600} 3.5) phase DMEM-MOPS cultures of Sakai and Sakai*rpoN*::*kan*. Following 2 h acid exposure in EG medium, plate counts for exponential-phase Sakai*rpoN*::*kan* were substantially greater than for Sakai, at 9.5×10^3 c.f.u. ml^{-1} and 10 c.f.u. ml^{-1}, respectively (n=3). Plate counts for stationary-phase Sakai*rpoN*::*kan* and Sakai did not differ, at 5.7×10^5 c.f.u. ml^{-1} and 5.5×10^5 c.f.u. ml^{-1}, respectively. Correspondingly, the survival rate (ΔV) of acid-challenged Sakai*rpoN*::*kan* grown to exponential phase in DMEM-MOPS, LBG-MOPS and LB-MOPS was significantly improved, at ΔV=−1.49, −1.73 and −0.82 log c.f.u. h^{-1}, when compared to Sakai, at ΔV=−4.48, −4.39 and −3.23 log c.f.u. h^{-1} (P<0.001). Since GDAR was altered in Sakai*rpoN*::*kan*, we wanted to determine if *rpoN* inactivation in other genetically distinct strains of *E. coli* O157:H7 also altered GDAR. As expected, exponential-phase DMEM-MOPS cultures of *E. coli* O157:H7 strains 93-111 and TW14359 did not survive well when inoculated into EG medium, at ΔV=−4.45 and −4.06 log c.f.u. h^{-1}, respectively. However, like Sakai*rpoN*::*kan*, ΔV values for 93-111*rpoN*::*kan* and TW14359*rpoN*::*kan* were significantly improved, at −1.49 and −2.04 log c.f.u. h^{-1} ($P\leqslant0.034$). Therefore, the inactivation of *rpoN* leads to increased acid resistance in *E. coli* O157:H7 by the GDAR system and *rpoN* allows for increased susceptibility to acid during exponential growth. Interestingly, examination of GDAR using DMEM-MOPS culture inocula of Sakai and Sakai*rpoN*::*kan* grown to OD_{600} 0.5, 0.75 and 1.0, when *gad* gene expression was greatest (Fig. 2), did not show significantly improved survival rates of either strain (data not shown). Complementation of Sakai*rpoN*::*kan* with a cloned version of the *rpoN* structural sequence to produce Sakai*rpoN*::*kan* pCR2.1(*rpoN*$^+$) reconstituted wild-type levels of acid susceptibility, at ΔV=−4.11 log c.f.u. h^{-1}, in exponential cultures of the complemented strain grown in DMEM-MOPS.

Since both RpoS and GadE act as regulators of GDAR, and many RpoS-regulated stress-resistance genes are altered following *rpoN* inactivation, we wanted to determine if inactivation of these genes in the Sakai*rpoN*::*kan* background would affect the expression of GDAR during exponential growth. Interestingly, GDAR in DMEM-MOPS, LBG-MOPS and LB-MOPS cultures was abolished in exponential-phase culture inocula of SakaiΔ*gadE rpoN*::*kan* and SakaiΔ*rpoS rpoN*::*kan*, where <10 c.f.u. ml^{-1} of both polygenic mutant backgrounds could be recovered following 0.5 h acid exposure in EG medium.

The effect of glutamate and different culture media on acid resistance in Sakai*rpoN*::*kan* was also examined. Glutamate is required for acid resistance by the GDAR mechanism and, as predicted, removal of glutamate from EG medium negatively affected the survival of exponential DMEM-MOPS culture inoculums of Sakai*rpoN*::*kan*, where <10 c.f.u. ml^{-1} were recovered following 0.5 h acid exposure. The survival of Sakai and Sakai*rpoN*::*kan* grown in LBG-MOPS compared to DMEM-MOPS, when inoculated into EG medium (pH 2), did not differ significantly. Growth in LB-MOPS, which does not contain glucose, did, however, significantly improve survival rates in EG medium for both Sakai and Sakai*rpoN*::*kan* (ΔV=−3.23 and −0.82 log c.f.u. h^{-1}, respectively), compared to survival rates when grown in LBG-MOPS (ΔV=−4.39 and −1.73 log c.f.u. h^{-1}, respectively, P<0.001) and DMEM-MOPS (ΔV=−4.48 and −1.49 log c.f.u. h^{-1} respectively, P<0.001).

The effect of *rpoN* inactivation on survival in a model stomach system was also determined. The model stomach is a complex *in vitro* system, containing multiple stressors such as bile salts, food matrix and acid, designed to emulate the complex *in vivo* stress conditions of the stomach (Bergholz & Whittam, 2007). We therefore examined cultures of Sakai*rpoN*::*kan* and Sakai grown to exponential phase (OD_{600} 0.5) in DMEM-MOPS, for survival in the model stomach system. Plate counts for Sakai*rpoN*::*kan* were significantly higher after 1 h (P=0.004) and 2 h (P<0.001) in the model stomach, whereas initial counts did not differ. After 1 h in the model stomach system, plate counts averaged 1.2×10^2 c.f.u. ml^{-1} for Sakai, and 4.5×10^5 c.f.u. ml^{-1} for Sakai*rpoN*::*kan*; and after 2 h, Sakai was reduced to 11 c.f.u. ml^{-1}, whereas counts for Sakai*rpoN*::*kan* averaged 3.8×10^5 c.f.u. ml^{-1}. Together these results suggest that *rpoN* inactivation leads to increased GDAR and survival in a model stomach system.

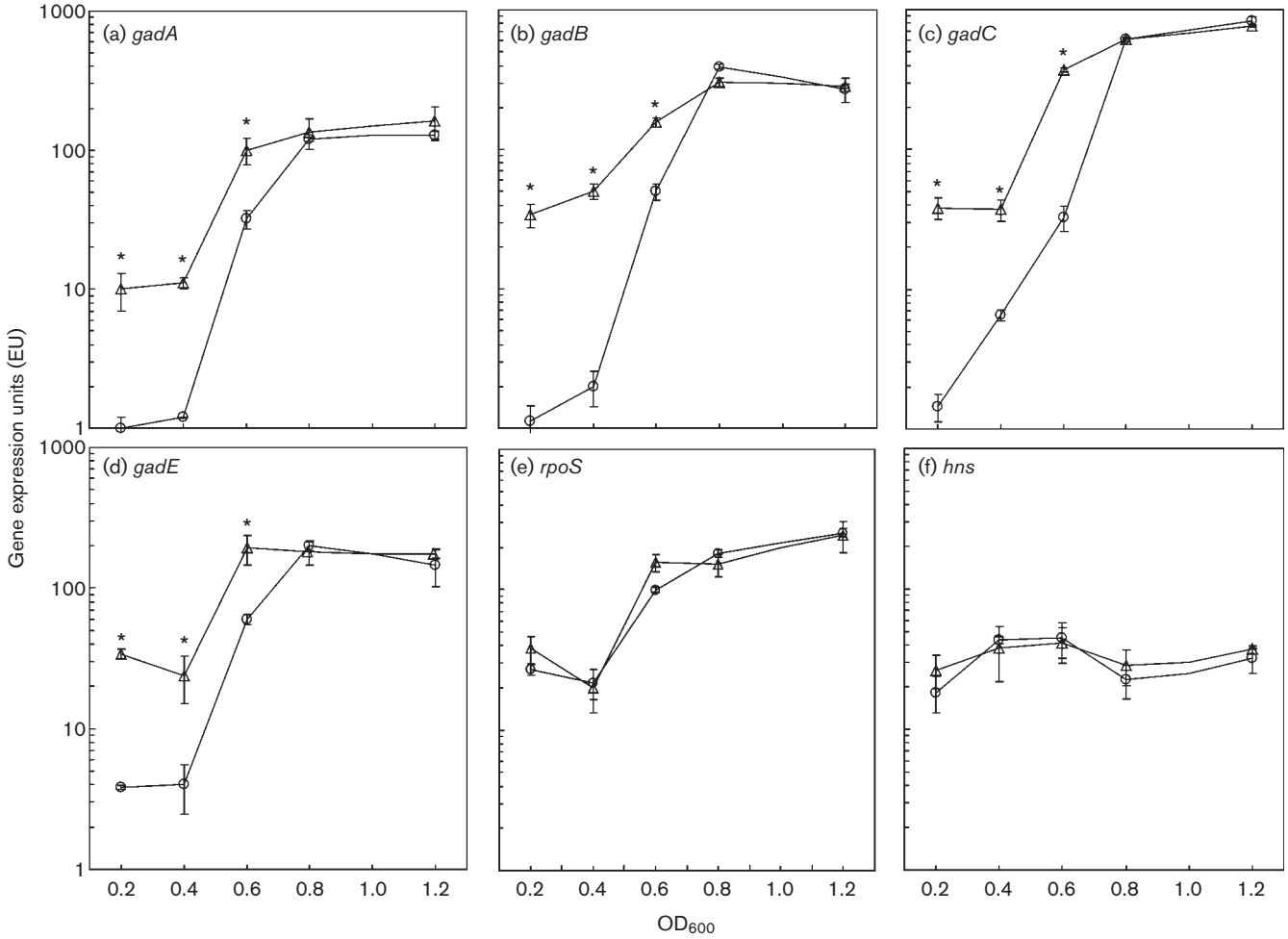

Fig. 2. Growth-phase analysis of *gad* genes, *rpoS* and *hns* expression by qRT-PCR. ○, Sakai; △, Sakai*rpoN* : : *kan*. Error bars represent SEM ($n=3$), and the asterisks denote significance by Welch's *t*-test ($P<0.05$).

DISCUSSION

There are 21 confirmed RpoN promoters in *E. coli* K-12 (Pallen, 1999; Reitzer & Schneider, 2001; Skibinski *et al.*, 2002; Vogel *et al.*, 2003), which specify the transcription of over 60 genes. This study identified 103 genes to be differentially expressed in the *rpoN*-inactivated *E. coli* O157 : H7 strain Sakai*rpoN* : : *kan*. The large majority of these are likely to be indirectly modulated through secondary regulators, as differential expression was observed in only 7/21 RpoN promoters, all seven of which specified the transcription of operons/regulators involved in the nitrogen regulatory (Ntr) response. This observation emphasizes the conditional nature of transcription from RpoN promoters, which are predisposed to idiosyncrasies in enhancer-binding protein (EBP) availability/activity. Interestingly, regulation by RpoN was growth phase dependent, with decreasing numbers of differentially expressed genes during transition into slower growth. As the abundance of RpoN in *E. coli* has been reported to be static (Jishage *et al.*, 1996; Jishage & Ishihama, 1997), this may reflect variability in the cellular level of various EBPs, or in the availability of ATP, which energizes EBP activity.

The LEE, encoding type III secretion system structural genes and regulators, was positively regulated by RpoN. This finding is particularly salient in light of recent studies demonstrating a role for RpoN in the regulation of a *P. aeruginosa* type III secretion system (Matz *et al.*, 2008). Type III secretion is essential to the A/E pathology of *E. coli* O157 : H7 and its expression is affected by numerous distally encoded factors, whose influence converges on the LEE-encoded activators Ler and GrlA (Spears *et al.*, 2006). In this study, *ler* was not observed to be altered in Sakai*rpoN* : : *kan*; however, *grlA* was downregulated. Thus, RpoN may affect LEE expression through indirect stimulation of *grlA*, the product of which can activate LEE transcription independent of *ler* (Russell *et al.*, 2007). RpoN may also regulate LEE through *rpoS*. We have already determined that *rpoS* is essential to increased GDAR in Sakai*rpoN* : : *kan* (see below), and RpoS is a known transcriptional regulator of the LEE. However, its

control of LEE expression has been reported to be through increased transcription of *ler*, not *grlA* (Iyoda & Watanabe, 2005; Laaberki *et al.*, 2006). RpoN could also regulate LEE through GadE. This protein has been reported to repress LEE expression independent of *ler* (Tatsuno *et al.*, 2003), and *gadE* expression was upregulated in Sakai*rpoN*::*kan* in this study.

Inactivation of *rpoN* was observed to affect the expression of stress resistance genes; most notably, *gad* genes of the GDAR system were shown to be upregulated in Sakai*rpoN*::*kan* during exponential growth. Moreover, upregulation of *gad* genes in Sakai*rpoN*::*kan* correlated with increased GDAR. This phenotype was validated in three distinct *E. coli* O157 : H7 strains representing major clonal lineages (Manning *et al.*, 2008), suggesting that regulation of GDAR by RpoN is conserved among *E. coli* O157 : H7.

Sakai*rpoN*::*kan* was dependent on the GDAR system for acid resistance. Both the exclusion of exogenous glutamate and deletion of the central regulator *gadE* in Sakai*rpoN*::*kan* abrogated GDAR. *gadE* and *gadX* were shown to be repressed by RpoN in microarray analysis; however, there are no RpoN-binding sites in the core promoters of these genes. RpoN may therefore alter *gad* gene expression through a secondary *trans*-/*cis*-acting factor(s). H-NS and RpoS are two important regulators of the GDAR system. H-NS represses *gad* transcriptionally through *gadA* and *gadX* (Giangrossi *et al.*, 2005; Tramonti *et al.*, 2006). Since *gad* transcription increases substantially in an *hns*-inactivated background (De Biase *et al.*, 1999), we initially hypothesized that RpoN may act indirectly to repress *gad* expression through *hns* activation. However, upregulation of *gad* genes in Sakai*rpoN*::*kan* was not associated with a decrease in *hns* transcript levels, and exponential cultures of an *hns*-inactivated strain of Sakai could not be recovered after 0.5 h of acid challenge (data not shown). Alternatively, RpoS activates *gad* transcription through the GadX/W circuit (Ma *et al.*, 2003). The results of this study reveal that the inactivation of *rpoS* in Sakai*rpoN*::*kan* abolishes exponential-phase GDAR, despite there being no difference in *rpoS* expression between Sakai*rpoN*::*kan* and Sakai (Fig. 2e). One hypothesis to explain this outcome is that reduced growth rates in *rpoN*-inactivated backgrounds (Supplementary Fig. S1) stimulate RpoS accumulation, which then leads to increased *gad* transcription. This argument is not well supported, however, as growth-rate attrition has only been shown to induce *rpoS* expression under conditions that increase generation times (g) to $>$140 min, or when cultures are grown under conditions of starvation (Gentry *et al.*, 1993; Lange & Hengge-Aronis, 1991). In this study, GDAR was observed in exponentially growing cultures of Sakai*rpoN*::*kan* (g=54 min) grown in nutrient excess. A more plausible hypothesis is that RpoN is controlling *rpoS* expression post-transcriptionally through an auxiliary regulator(s), of which at least ten have been described for *rpoS* (Hengge-Aronis, 2000). As RpoS is known to regulate several mechanisms of acid resistance in *E. coli* (Foster, 2004), interplay between RpoN and RpoS could have a broad impact on the acid resistance phenotype and on stress resistance in general. Interestingly, an RpoN-RpoS regulatory pathway has been recently described in the enteric pathogen *S. enterica*, and in the aetiological agent of Lyme disease, *Borrelia burgdorferi*. In *S. enterica*, RpoN-RpoS have been determined to co-regulate O antigen LPS production through *rfaH* (Bittner *et al.*, 2002, 2004), and in *B. burgdorferi*, RpoN-RpoS regulation has been shown to be essential to transmission and pathogenesis through control of membrane lipoproteins OspC and DspA (Boardman *et al.*, 2008; Hubner *et al.*, 2001; Yang *et al.*, 2005). Collectively, these findings testify to the importance of RpoN-RpoS-directed regulation in bacterial stress resistance and pathogenesis.

Conclusions

In this study, *rpoN* inactivation in *E. coli* O157 : H7 resulted in the differential expression of 103 genes by microarray analysis, including acid resistance and LEE genes, which are essential to transmission and pathogenesis. GDAR genes were upregulated in Sakai*rpoN*::*kan* during exponential growth, which correlated strongly with increased acid resistance by the GDAR mechanism, and in a model stomach system. Acid resistance by the GDAR mechanism in Sakai*rpoN*::*kan* during exponential growth required intact *rpoS* and *gadE* genes. Genes encoded throughout the LEE were downregulated in Sakai*rpoN*::*kan* during exponential growth. This included the LEE regulator *grlA*. Together these results suggest that RpoN is an important growth-phase-dependent regulator of acid resistance and LEE expression. Future research will explore further the mechanism by which RpoN directs GDAR expression, its regulatory dependence on *rpoS*, and the full contribution of *rpoN* to stress resistance and virulence in *E. coli* O157 : H7.

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