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BIOFILM FORMATION OF *Salmonella enterica* AND THE CENTRAL ROLE OF RPOS SIGMA FACTOR IN STRESS RESISTANCE

WAN ZAWIAH WAN ABDULLAH[1,2*], BERNARD M. MACKEY[1] and KIMON-ANDREAS G. KARATZAS[1]

[1]*Department of Food and Nutrition Sciences, University of Reading, P.O. Box 226, Whiteknights, Reading RG6 6AP, UK*
[2]*School of Food Science and Technology, Universiti Malaysia Terengganu, 21030, Kuala Nerus, Terengganu, Malaysia*
[*]*E-mail: wzawiah@umt.edu.my*

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ABSTRACT

Non-typhoidal *Salmonella* is considered as the leading cause of foodborne illness and it has been associated with high-profile outbreaks in many groups of foods. This work examines the contribution of phenotypic properties related to survival (biofilm formation) and how these are linked with the genetic and functional variability of *rpoS* gene and RpoS status respectively in *Salmonella enterica.* The test strains were *Salmonella* serovars Anatum, Enteritidis (466), Enteritidis (496), Hadar, Heidelberg, Montevideo, Newport and Virchow and two Typhimurium strains previously characterised as either RpoS-positive or RpoS-negative. RpoS status was found to affect biofilm formation. The capability of *Salmonella* to resist stress and survive under unfavourable conditions can vary between strains. We confirmed that the two strains that were previously sensitive to various stresses harboured significant mutations in the *rpoS* gene. The *rpoS* sequencing not only confirmed a link between RpoS and biofilm formation, but it also revealed a link with differences in the utilisation of carbon sources. The RpoS-negative phenotype was linked with an increased growth under different carbon sources suggesting that a functional RpoS is a burden for growth which is in agreement with the SPANC hypothesis.

Key words: *Salmonella enterica*, biofilm, RpoS

INTRODUCTION

Salmonella is good at forming biofilms, which provide protection against low pH, desiccation, host immune responses and antimicrobial agents (Steenackers *et al.*, 2012). Flagella are involved in biofilm formation but also play several other important roles in the survival of *Salmonella* including chemotaxis and competition for nutrients, adherence to and invasion of gut epithelial cells, and binding to cholesterol in gallstones within the gall bladder. Given the wide range of distinct cellular properties involved in competition and survival, it would be reasonable to expect that exposure to different stresses would select for variants with phenotypes, giving particular advantages for specific environments or conditions and there is now increasing evidence for considerable variation among strains in resistance to heat, acid, desiccation, and biofilm formation (Lianou & Koutsomanis, 2013).

In order to increase the likelihood of survival against stress conditions, *Salmonella* has evolved several mechanisms, of which the most important is RpoS (Jordan *et al.*, 1999). RpoS (σ^s) is usually found in Gram-negative bacteria (*Salmonella*, *Escherichia coli*, *Vibrio* spp., and *Pseudomonas*) and plays a role under sub-lethal stresses, for instance in low pH (Lee *et al.*, 1995), osmotic shock (Hengge-Aronis, 1996), as well as entry into the stationary phase (Kolter *et al.*, 1993).

Several researches discovered that RpoS activates the transcription of 100 genes and operons that form part of the bacterial virulence and defence mechanisms (Patten *et al.*, 2004). Interestingly, the stress resistance depends on RpoS, while on the other hand, the nutritional competence depends on

* To whom correspondence should be addressed.

house-keeping sigma factor RpoD. According to Ferenci (2005), Self-Preservation and Nutritional Competence, also known as the SPANC balance, depends on works with spontaneous *rpoS* mutants appearing frequently in *E. coli*. In addition, King *et al.* (2004) demonstrated that the SPANC balance is the inverse relationship between self-protection and nutritional competence.

Stress-sensitive *rpoS* mutants are surprisingly common amongst natural bacterial isolates, especially *Escherichia coli* and *Salmonella*. The frequency of these mutants can be explained because they have a growth benefit under certain conditions of limited nutrients and are rapidly selected in aged batch cultures or nutrient-limited chemostat cultures. In *Salmonella* and *E. coli*, there is thus a trade-off between nutritional competence and survival potential (Ferenci, 2005), and this is a powerful mechanism giving rise to phenotypic heterogeneity.

MATERIALS AND METHODS

Salmonella enterica strains

Table 1 shows the *Salmonella* strains that are utilised in the present study. The cultures were maintained at -70ºC in cryovial tubes (Greiner Bio-One Ltd., Stroudwater Business Park, GL10 3SX UK). The *Salmonella* Typhimurium strains were supplied by Professor T.J. Humphrey, Institute of Infection and Global Health, University of Liverpool, UK, and the remaining strains by Mrs. Linda Ward of the Laboratory of Gastrointestinal Infections, Public Health England, Colindale, UK.

Biofilm formation

10 mL of Low Salt LB in a 25 mL universal bottle was inoculated with one loopful of overnight cultures, followed by being incubated with shaking at 25°C in a period of 4 days. Pellicle formation was recorded before discarding the culture medium. Biofilm adhering to the glass was recorded before and after staining with an aqueous crystal violet solution of 0.1% (w/v).

The following quantitative method was used to test biofilm formation on polystyrene plastic: 200 mL of overnight culture cultures were put into 96 microwell plates (flat bottom) together with a blank and a control (LB broth without salt). The 96 flat bottom microplates were incubated at 25°C. After 4 days, the liquid cultures were discarded and the plate that has been used for biofilm formation was washed twice with distillled water using multi pipette. The biofilm formation of ten different strains and serovars of *Salmonella* were stained using 0.1% aqeous crystal violet stain and were left for about 20 minutes. The adherent stained biofilms were dissolved with 95% ethanol and the optical density was measured at 620nm (OD_{620nm}) at one to two min in a Tecan Sunrise microplate reader (Tecan UK Ltd, Theale, RG7 5AH UK) operated by the Magellan software. A higher reading reflected a stronger biofilm formation.

Sequencing the *rpoS* gene

The Instagene Matrix (Bio-Rad) was used to extract the template DNA from the cultures in accordance with the manufacturer's instructions. The primers that have been utilised for the *rpoS* gene sequencing are shown in Table 2. The primers were synthesised by Sigma-Aldrich and PCRs were run on a Bio- Rad MJ Mini Personal Thermal Cycler (Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire). The isolation of PCR products were performed via electrophoresis on an agarose gel of 2% (w/v) containing ethidium bromide (5 mL per 30 mL gel). Electrophoresis was performed for 45 minutes at 100 V. Based on the manufacturer's instructions, the gels were visualised under UV and PCR products were extracted from the gel and cleaned via the MinElute PCR Purification Kit (Qiagen UK, Crawley, UK). Sequences were generated from the amplified PCR products via

Table 1. *Salmonella enterica* serovars used in this study

Serovar	Strain number	Isolated from	Source or reference
Anatum	S180332	Dried Food	Laboratory of Enteric Pathogens, Public Health Laboratory Service. Colindale, London NW9 5EQ[1]
Heidelberg	S172457	Chicken	
Montevideo	S182788	Pet food	
Newport	S186166	Peppercorns	
Enteritidis	P518466	Human	
Enteritidis	P518496	Human	
Virchow	P518634	Pet food	
Hadar	P518721	Meat	
Typhimurium DT 104	Strain 10	Human	Jorgensen *et al.* 2000 *Microbiology*, **146**: 3227-3235
Typhimurium DT 104	Strain 30	Bovine	

[1]Now Public Health England, Microbiological Services

Table 2. Primers used for sequencing the *rpos* gene

Primer	Nucleotide sequence (5' – 3')
RPOS3*	CCTTGCCCGGGCTGTGCCGATGCAC
RPOS5*	CGG AATTCTTATTATCATCAA ACATAAC
RPOS3	TCGCATGACGCAAAAAGATA
RPOS3	TCCACAAGCGTTTCGTATGAC
RPOS5	GCGACTCAGCTTTACCTTGG

*denotes sequencing primers from Jorgensen *et al.* (2000). All primers supplied by MWG Biotech, London, UK.

Source Bioscience, Department of Biochemistry, University of Oxford.

Substrate utilisation

(a) Cell suspensions preparation

The strains of *Salmonella* were grown on BUG+B agar plates by streaking the bacteria for isolated colonies and allowing them to grow overnight at 30–37°C. A second subculture was made if the cells were streaked from a frozen culture stock. By using a disinfected swab, the cells were removed from the BUG+B agar plate. Afterwards, they were relocated into an aseptic capped tube comprising 16 mL IF-0 suspending medium (Biolog). Subsequently, the swab is used to stir the cell suspension so as to gain a uniform suspension. The suspension was checked for turbidity and the cells were later added to obtain 42% T (transmittance) in the Biolog Turbidimeter. After that, a cell suspension containing 15 mL of 42% T was added to the vial consisting of 75 mL of IF-0+ tetrazolium redox dye (1:5 dilution).

(b) Inoculation of Phenotypic Microarrays PM 1 and PM 2

22 mL of the 85% T cell suspension prepared in two steps was transferred into a sterile reservoir. PM1 and PM2 were inoculated about 100 micro-litres with this cell suspension.

(c) Incubation and data collection

The worksheet data was entered into the OmniLog Software. OmniLog was loaded with PM1 and PM2 plates. All PMs were incubated at 37ºC for 24 to 48 hours. After 48 hours, the plates were removed from OmniLog and stored at 4ºC. The data was collected for analysis.

Data analysis

The data from replicate experiments was analysed by using one-way ANOVA. All experiments were performed independently on three separate occasions. The findings are depicted as average values, whereas error bars are representing as standard deviation.

RESULTS

Biofilm formation

Biofilm formation on glass was measured qualitatively by incubating strains in the LB broth without sodium chloride at 25ºC with shaking. Eight of the ten test strains consistently formed good biofilms on the glass surface (data not shown). Biofilm was absent in the two RpoS negative strains *Salmonella* Heidelberg and *Salmonella* Typhimurium strain 10. Serovars that showed good biofilm formation also produced obvious pellicles on the liquid surface and showed pronounced sedimentation of cells in the culture. The cultures of RpoS-negative strains had uniform turbidity and showed no sedimentation. The biofilm formation was also assessed after growth in polystyrene microwell plates (see Methods). Table 3 shows the mean optical densities of stained biofilms. The results have a great likeness to the findings obtained after the growth in glass bottles with no biofilm formation in serovars Heidelberg and Typhimurium 10.

Table 3. Mean optical densities (at 620nm) of stained biofilms of *Salmonella enterica* strains after growth in polystyrene microwell plates at 25ºC

Serovar	Optical density	Standard deviation	Rank
Typhimurium (30)	0.38	0.05	1
Hadar	0.32	0.03	2
Virchow	0.37	0.16	3
Anatum	0.34	0.09	4
Enteritidis (496)	0.33	0.06	5
Newport	0.27	0.06	6
Montevideo	0.20	0.03	7
Enteritidis (466)	0.15	0.08	8
Heidelberg	0.03	0.01	9
Typhimurium (10)	0.02	0.01	10

Genetic analysis

Following the *rpoS* genes of the strains, it is revealed that *Salmonella* Typhimurium strain 10 had G to T transversions at nucleotide number 99, in which the GAG triplet codon is changed and the glutamic acid is specified to a TAG amber nonsense stop codon at position 33 in the amino acid sequence of the protein. Furthermore, *Salmonella* Heidelberg had an insertion of a T at nucleotide number 640, causing a frameshift mutation and leaving intact only the first 213 amino acids in the sequence of the protein.

Substrate utilisation

The Phenotype Micro Array Technology of Biolog, Inc (Hogward CA) measures the cell respiration as a function of time in numerous micro wells simultaneously by colorimetric detection of purple coloured formazan derived from tetrazolium dye, following the metabolic activity of cells that corresponds to the intracellular reducing rate by NADH. The carbon substrates that are discovered in distinctive microtitre plates in the Biolog experiment are separated into the substrate guilds. The chemical guilds are amine/amides, amino acids, carbohydrates, carboxylic acid, polymers, and other compounds.

The test was performed on 192 carbon sources using PM1 and PM2 Biolog Microplates. In PM1 Microplate, *Salmonella* grew on 67 carbon sources out of 96 contained on plates. Surprisingly, in PM2 Microplate, the *Salmonella* strains were able to utilise only 6% of carbon sources contained on that microplate (data not shown).

From the Biolog experiments, it is clear that the RpoS-negative serovars grew significantly better that the RpoS-positive ones utilising different carbon sources on PM1 and PM 2 Microplates. The rank order analysis of the maximum OmniLog unit on 73 substrates (Table 4) demonstrated clearly that the RpoS-negative serovars *S*. Typhimurium (10) and *S*. Heidelberg were the fastest growing serovars on these carbon sources (mean Maximum OmniLog unit = 129 and 128, respectively), followed by RpoS-positive serovars such as *S*. Anatum. Finally, *S*. Newport was ranked last as the most inefficient strain in utilising the different carbon sources (mean Maximum OmniLog unit = 86).

DISCUSSION

It is assumed that *S. enterica* strains with strong biofilm formation have a comparatively high food safety risk as a result of the survival probability in food-processing environments, and as a consequence, this has given rise to their virulence potential. Smirnova *et al.* (2010) and Steenackers *et al*. (2012) both demonstrated that the structural components of *S. enterica* biofilms comprise surface proteins (familiarly recognised as biofilm associated protein), curli and other fimbriae, cellulose, flagella, colonic acid, fatty acids, and anionic o-antigen capsule. Cellulose and curli fimbriae (previously referred to as aggregative fimbriae) are the basic components of the extracellular polymeric matrix of *Salmonella* biofilms. These components results in the production of greatly hydrophobic system of tightly packed cells (Smirnova *et al*., 2010; Gerstel & Römling, 2001; 2003; Steenackers *et al*., 2012).

Interestingly, the role of RpoS has been linked to biofilm formation. Environmental factors (for example, temperature, nutrients and starvation, osmolarity, pH) give impact on *Salmonella* biofilm formation by performing upon CsgD expression via RNA polymerase sigma factor RpoS, small nucleotides, and multiple transcriptional factors. The sigma factor RpoS dominates other sigma factors when cells of *Salmonella* enter the stationary growth phase (Hengge-Aronis, 2002). Curli subunit gene *csgD* is plays a vital role in biofilm formation and is a master transcriptional controller for producing the extracellular matrix of biofilm. Gibson *et al*. (2006) showed that the biofilm's resistance to stress factors greatly decreased after the knockout of the *csgD*, *csgA*, and *bscA* genes.

Table 4. Rank order analysis of Maximum OmniLog unit

Serovar	RpoS	Mean Max OmniLog Unit	Rank order
Typhimurium (10)	negative	129	1
Heidelberg	negative	128	2
Anatum	positive	101	3
Virchow	positive	96	4
Hadar	positive	95	5
Montevideo	positive	94	6
Enteritidis (496)	positive	92	7
Typhimurium (30)	positive	92	8
Enteritidis (466)	positive	91	9
Newport	positive	86	10

In this study, there is a relationship between RpoS and biofilm formation. All RpoS-positive serovars that produced a rdar (red, dry, rough) morphotype from the previous study (data not shown) also formed a biofilm. On the other hand, the RpoS-negative serovars (*S*. Heidelberg and *S*. Typhimurium 10) that formed white, smooth colonies consistently produced no biofilm. Römling *et al.* (1998) demonstrated that the deletion of *agfD* resulted in the rdar morphotype transforming to a white and smooth colony, and such mutans deprived of all types of multicellular behaviour. Furthermore, the authors noted that the process of transcribing *agf* operons necessitated *rpoS* and *OmpR*. This coincides with our results where biofilm formation was correlated with RpoS phenotype and colony morphology.

Stress sensitive *rpoS* mutants are surprisingly common amongst natural bacterial isolates, especially *Escherichia coli* and *Salmonella* (Notley-McRobb *et al.*, 2002). In our study, we investigated the existence of possible mutations in the *rpoS* of the serovars used in this study. As previously described by Jorgensen *et al*. (2000) that strain mutation in *S.* Typhimurium (10) caused loss of function, this study showed that *S.* Heidelberg also contained a mutation in the *rpoS* gene, possibly resulting in function deficiency as well. Sequence variation does occur and that these changes of RpoS-positive serovars at the protein level are mainly located at the centre (region 3) of the coding region. The location of the protein changes found in *Salmonella* indicated that certain areas of the gene remain conserved within the subspecies of *S. enterica*. These regions are probably highly important for the proper function of the protein.

RpoS is an important cell constituent that facilitates survival under adverse conditions, therefore it is to be expected that some sections of the gene may be conserved. Sutton *et al.* (2000) suggested that mutations in the *rpoS* gene may be distributed randomly through the coding sequence. In our study, we demonstrated that the mutations in *rpoS* of *S*. Heidelberg were unique in comparison to other *Salmonella enterica* and other species.

A number of laboratory strains of *Escherichia coli*, for instance, strains AB1157, RM4606, and JC7 623, comprise mutations at nucleotide C to T and result in TAG amber nonsense codons similar to the *rpoS* gene in DT 104 strain. In addition, *E. coli* strains CL1010 and JK12010 as well contain mutation C to T change at codon 33 of *rpoS* (Visick & Clarke, 1997). In our study, we confirmed the mutation in *rpoS* of *S*. Typhimurium strain 10 identified by Jorgensen *et al.* (2000).

Our results confirmed a well-established knowledge that a functional RpoS protein is necessary for stress resistance affecting the general physiology and behaviour of the bacterium. It was demonstrated in a previous study (Humphrey *et al*., 1996) that strains containing mutations in the RpoS gene, for example, PT4 strains, were seldom discovered in the tissue of contaminated birds. Moreover, according to Williams *et al.* (1998), there was no significant difference between stress-sensitive strains from faecal material and stress-resistant strains in muscle tissue. This finding showed a complex correlation between the RpoS expression, pathogenicity and stress tolerance. However, it is highly interesting that although these *rpoS* mutations do not promote survival under stressful conditions they seem to appear very often.

The most convincing justification for the appearance of the *rpoS* mutants in *E. coli* is the trade-off between nutritional competence and survival potential; and this is a powerful mechanism giving rise to phenotypic heterogeneity (Ferenci, 2005). To investigate if this concept also applies in *Salmonella*, we compared the RpoS-negative and –positive serovars for their utilisation of carbon sources. It is shown that this concept also applies in *S. enterica.* This is clear since the RpoS-negative serovars; *S*. Typhimurium (10) and *S*. Heidelberg were significantly more efficient in utilising a wide range of different carbon sources. Similarly, Weber *et al.* (2005) demonstrated that RpoS-positive strains were more resistant to severe treatments but they grew slower under a variety of conditions. King *et al.* (2004) demonstrated that RpoS-positive strains are less competitive than RpoS-negative strains in terms of environments with limited glucose.

All the above suggest that RpoS-negative serovars, by losing their ability to survive in adverse conditions, gain a competitive advantage under nutrient limitation. Metabolic genes, among other genes, that are engaged in nutrient uptake are in some reverse manner under the control of RpoS. It has been suggested that this is the results of sigma factor competition. According to Maeda *et al*. (2000), it is found that RNA polymerase core components in *E. coli* K-12 and RpoD (house-keeping sigma factor) were relatively constant. Nevertheless, there is a difference in terms of concentration among the other sigma factors stimulated by environmental signals. Due to the competition for core RNA polymerase, the elevated levels of RpoS resulted in the increased expression from several promoters and the decreased expression from RpoD promoters. Since transporter genes were four- to seven-fold down-regulated by RpoS antagonism, they were less able to compete for the low nutrient level (Notley-McRobb *et al.*, 2002). Moreover, it is worth mentioning that a few authors noted the poor dietary range of high-RpoS strains, which metabolise less substrates and tend to grow considerably more slowly on sources of carbon, for

instance, succinate, acetate, sorbitol, melibiose, glycerol phosphate, and asparagine (Chen *et al.*, 2004; King *et al.*, 2004).

CONCLUSION

It can be concluded that the study provide new insights into the mechanisms, whereby *Salmonella* is capable to survive stresses that are widely used in the manufacturing and safe storage of food products. To this point, the survival strategies and stress responses of *Salmonella* can provide important knowledge on the pathogenesis and epidemiology of these bacteria. The nutrient substrate utilisation profiles determined from *Salmonella* isolates are useful references of the substrates used by individual strains and they are related to the ability of *Salmonella* to persist under severe treatments. Interestingly, the *rpoS* mutation has benefits to *Salmonella* under nutrient limitation.

In addition, this study has implications for predicting the survival of *Salmonella* under adverse conditions and also in the choice of strains in food safety challenge studies used to provide data for risk assessments.

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