Canadian Journal of Microbiology
Revue canadienne de microbiologie

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Journal:	<i>Canadian Journal of Microbiology</i>
Manuscript ID	cjm-2017-0059.R1
Manuscript Type:	Note
Date Submitted by the Author:	16-May-2017
Complete List of Authors:	Ganguly, Arpeeta; University of Delaware, Animal and Food Sciences Joerger, Rolf; University of Delaware, Animal and Food Sciences
Keyword:	Salmonella gene, Salmonella sulfatase, acid sulfatase, sugar sulfate ester, tyrosine sulfate

Sugar sulfates are not hydrolyzed by the acid-inducible sulfatase AslA from *Salmonella enterica* Enteritidis Nal^R and Kentucky 3795 at pH 5.5

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ABSTRACT

The open reading frames (ORFs), SEN0085 and SeKA_A4361, from *Salmonella enterica* Enteritidis Nal^R and Kentucky 3795, corresponding to the acid-inducible sulfatase gene, *asIA* from *S. e* Typhimurium, were previously suggested by microarray analysis to be differentially expressed under acid conditions. However, growth and enzyme activity tests in the present study demonstrated that both wild type strains exhibited sulfatase activity with 4-nitrophenyl sulfate and 5-bromo-4-chloro-3 indolyl sulfate at pH 5.5. The acid sulfatase does not appear to be involved in sugar sulfate, tyrosine sulfate and 4-hydroxy-3-methoxyphenylglycol sulfate, heparin or chondroitin sulfate hydrolysis at pH 5.5. Adhesion and invasion assays did not reveal differences between the serotypes and their corresponding *asIA* deletion mutants. Thus the role and substrate(s) of AsIA, a protein unique to *Salmonella* and encoded in all sequences *Salmonella* strains, remain elusive.

Keywords

Salmonella; acid sulfatase; sugar sulfate;

Since 1998, *Salmonella enterica* serovar Kentucky has been isolated from meat and poultry products in the United States far more frequently than other serovars. For example, in 2013, 28% of all *Salmonella* isolates from these sources were of the Kentucky serovar, followed by *S. e.* Montevideo (10%) and *S. e.* Enteritidis (9.4%) (USDA 2013). A comparison of phenotypic traits of serovar Kentucky and other poultry *S. e.* isolates revealed that this serovar was more sensitive to exposure to pH 2.5 than most other *Salmonella* isolates (Joerger et al. 2009); however, when growth of *S. e.* Kentucky 3795 in medium adjusted to pH 5.5 with HCl was compared to that of *S. e.* Enteritidis Nal^R, the Kentucky strain was able to grow better at this pH than the Enteritidis strain. Microarray-based gene expression analysis of the two strains exposed to HCl-induced pH 5.5 revealed few distinct differences between the gene expression patterns of the two isolates, but a prominent difference involved the *hdeB*-like gene that appears not to be expressed in the Kentucky isolate (Joerger and Choi 2015). Among other genes or open reading frames that showed differences in expression was the gene previously designated *aslA*. This gene, corresponding to locus SEN0085, STM0084 and SeKA_A4361 in *S. e.* Enteritidis, Typhimurium and Kentucky, respectively is predicted to encode a sulfatase-like protein. Upon exposure to medium with an HCl-induced pH of 5.5, the Kentucky strain responded with a more than 5-fold increase in mRNA expression signal, whereas essentially no change in the corresponding signal for the Enteritidis strain was observed (Joerger et al. 2012). Thus, it is of interest to corroborate these results with data from enzyme activity and growth data.

The role of *aslA* in *Salmonella*'s interaction with hosts or the environment is currently not well understood. Screening of transposon mutant libraries indicated that insertions in STM0084 did not have a strong impact on competitiveness of the mutant strains in in-vivo assays with mice injected with mutants and wild type (Chaudhuri et al. 2009), but when chickens, cattle and pigs were infected orally with the transposon mutants, mutants with insertions in STM0084 were less competitive (Chaudhuri et al. 2013). This observation suggests a possible role of this gene in host interactions in the GI tract. Das et

al. (2013) deleted *as/A* in *S. e. Typhimurium* LT2 and demonstrated that it is expressed under mild acid conditions, but not at pH 7. Interestingly, the authors also showed that the enzyme that could potentially yield a carbon and/or sulfur source, was not regulated by sulfate or glucose.

In an attempt to further our understanding of the role of *as/A* and its potentially differential regulation in *S. e. Enteritidis* Nal^R and Kentucky 3795, the gene was deleted in the current study and the utilization of sulfate ester compounds by the mutant and wild-type strains was assayed. The competitiveness of the mutants versus the wild-type strains in cell-invasion assays was also determined. The genes corresponding to SEN0085 in *S. enterica* Enteritidis Nal^R and SeKA_A4361 in *S. enterica* Kentucky 3795 were replaced with kanamycin-resistance cartridges as previously described (Joerger and Choi 2015) by utilizing Lambda red-mediated gene replacement. The 3'

TAAATGAATATGTGATAAAGTTACTGACTACAGGTATAATAATGTGTAGGCTGGAGCTGCTTC 5' and 3'

ACACATATTCTTACCCGGAAGG 5' forward primers and 3'

TAAATGAATATGTGATAAAGTTACTGACTACAGGTATAATAATGTGTAGGCTGGAGCTGCTTC 5' and

3'GCCTTAAATCGCTGACCAATCG5' reverse primers were used for mutagenesis and to confirm the success

of the procedure respectively. The wild-type and mutant *S. e.* strains were then grown and maintained

on tryptic soy agar (TSA) and TSA with kanamycin (40 µg/mL), respectively for growth studies with

different potential sulfur sources. Das et al. (2013) suggested that the enzyme may have a role in

utilization of sulfated glycans in the host. The mouth and digestive tracts are rich in sulphomucins,

predominantly N-acetylglucosamine 6-sulphate, galactose 6-sulphate, galactose 3-sulphate and

galactose 4-sulphate (Roberton and Wright 1997). While sulphomucins limit mucin degradation by

mucin-degrading bacterial enzymes and protect the underlying luminal surface, they are also a target for

certain bacteria that hydrolyze the protective layer of sulfated glycans and establish infection. The wild-

types and mutant strains of *S. e. Kentucky* 3795 and *S. e. Enteritidis* Nal^R strains were inoculated into 3-

[N-morpholino] propane sulfonic acid (MOPS) Minimal Medium (Neidhardt et al. 1974) in which the

MOPS component was replaced by 2-[N-morpholino] ethane sulfonic acid (MES) at the same molarity and glycerol at 50 mM was the sole carbon source.

Das et al. (2013) had determined AlsA activity with cells grown at pH 5.5 in the presence of MOPS. MES was chosen in the current study as it exhibits better buffering capacity than MOPS at this pH. When sulfatase activity in MES medium buffered at pH 5.5 was compared to that obtained with cells grown in MOPS medium with the same starting pH, it was observed that the activity of cells grown in MES medium was lower than that of cells grown in MOPS medium (data not shown). Measurements of the pH of the cultures showed that MOPS medium was unable to maintain the initial pH and the pH dropped to below 4 after 24 h. This observation suggested that expression of *alsA* could possibly be higher at a pH lower than 5.5. Therefore, sulfatase activity assays were done with MES medium pH 3.75 to 6.5 in increments of 0.25 pH units. The results for sulfatase activity after 24 h are shown in Table 1 and these data indicate that the activity is highest at a pH of 4.5 to 4.75. Therefore, growth studies with sulfate-starved wild-type cells and potassium 4-nitrophenyl sulfate (Sigma) as sulfate source were carried out at pH 4.5, 4.75, 5 and 5.5. Growth was considerably higher at pH 5.5 than the other pHs tested (data not shown). Therefore, growth studies with different sugar sulfates were carried out at this pH. For these studies, cells were first grown for 24 h at 30 °C with shaking in MES medium without added sulfate and with a pH adjusted to 7 with KOH. The cells were pelleted by centrifugation, re-suspended in sulfate-free MES-containing minimal medium with added glycerol at pH 5.5 and cultured for an additional 24 h under the same conditions. Cells were harvested by centrifugation and resuspended in sulfate free MES-containing medium with added glycerol, pH 5.5 at an initial OD₆₀₀ of 0.05. Ninety-μL aliquots of the cell suspensions were pipetted into wells of a 96-well microtiter plate and 10 μL of either 1 mM K₂SO₄, sterile deionized water or 1 mM of filter-sterilized sulfur sources in deionized water was added. The following sulfur sources were used: *D*-galactose 4-sulfate (Sigma); *D*-mannose 6-sulfate (Sigma); *D*-

galactose 6-sulfate (Sigma); chondroitin sulfate (Alfa Aesar); heparin sodium salt (Sigma-Aldrich); potassium 4-nitrophenyl sulfate (Sigma).

Growth in the presence of the above mentioned sole source of sulfate was monitored by reading the optical density with a plate reader (Biotek Instruments Inc.) at 600 nm. Each culture condition was replicated in four wells and the average of the readings from the four wells were calculated. The difference between the OD₆₀₀ of the wells containing 1 mM K₂SO₄ and those without added sulfate was set at 100% and compared to the difference between the OD₆₀₀ obtained by the culture with the different organic sulfur sources and the OD₆₀₀ of the cultures without added sulfate. Neither the wild types nor mutant strains of *S. e. Kentucky 3795* and *S. e. Enteritidis Nal^R* showed any difference (Tuckey's test; $p < 0.05$) in growth. Thus *asfA* does not play a role in the utilization of the tested sulfated glycans as suggested by Das et al. (2013). In contrast, the wild-type strains were able to utilize 1 mM 4-nitro phenyl sulfate as a sulfur source nearly to the extent as 1 mM K₂SO₄ (Fig. 1).

Sulfated phenolic compounds are not uncommon in nature and can also be found in proteins in the form of tyrosine-o-sulfate. One can speculate that this compound could be used by *Salmonella* as a source of sulfur and the amino acid, or that the hydrolysis of the ester bond could modulate certain cellular activities. For example, it is known that tyrosine sulfatation of gastrin, a hormone involved in regulating gastric acid secretion and growth of the mucosa in the gastrointestinal tract modifies the activity of the hormone (Jensen et al 1981). Tyrosine sulfate was synthesized for this study according to Liu et al. (2009). The compound was resolved on a Waters preparative HPLC system equipped with mass spectrometric detection (negative ionization) using XBridge® BEH Prep OBD™ Amide (19 x 50 mm) column, 5 µm pore-size, with a linear gradient of 5 % to 40 % water in acetonitrile over 5 minutes), when added as sole sulfur source, the synthesized tyrosine sulfate did not lead to distinguishable levels of growth by mutant and wild type strains (Tuckey's test; $p > 0.05$). It is possible that an enzyme other than that encoded by *asfA* is responsible for the release of some sulfur, by it is also possible that the tyrosine

sulfate preparation contained some free sulfate (Fig. 1). Free sulfate could also be the reason for the minimal growth on heparin and chondroitin sulfate.

Another group of sulfated compounds arises from the metabolism of catecholamines. These compounds play a role in pathogenesis, including that of *Salmonella* (Spencer et al 2010). Growth of sulfate-starved wild and *asIA* mutant strains of *S. e.* Enteritidis and *S. e.* Kentucky in the presence of 4-hydroxy-3-methoxyphenylglycol sulfate potassium salt (Sigma), a metabolite of norepinephrine degradation, did not reveal any difference, indicating that this compound is not a substrate for *AsIA* (Fig 1). It is possible that only the actual hormone sulfate esters are recognized by the enzyme, but these esters were not available for this study.

To determine if the microarray-based differences in transcript accumulation of *asIA* mRNA between *S. e.* Enteritidis and *S. e.* Kentucky were reflected in the sulfatase activity at pH 5.5, the activity was monitored in the corresponding wild-type and *asIA* mutant strains as described by Das et al. (2013); except that the cells were grown in minimal medium with MES buffer and glycerol as carbon source. Activity on 5-bromo-4-chloro-3 indolyl sulfate was visualized on the respective minimal media solidified with 1.5% agar as production of a blue color. Sulfatase activity was observed with both wild-type strains with 4-nitrophenyl sulfate (Table 1) and 5-bromo-4-chloro-3 indolyl sulfate (data not shown), under appropriate acidic conditions. The sulfatase activity was generally slower to develop in the Enteritidis strain (data not shown), presumable because this strain is more affected by exposure to pH 5.5 (Joerger et al. 2009).

In vivo studies by Chaudhuri et al. (2013) in pigs, cattle and poultry and Das et al. (2014) in zebrafish have indicated the importance of *S. e.* Typhimurium *asIA* in causing infection/survival within animal cells. Infection of confluent RAW 264.7 cells obtained by growing the cells at 37°C and 5% CO₂ for 48 h in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Inc., Manassas, VA) containing 4.5 g/L of glucose, L-glutamine, and sodium pyruvate amended with fetal bovine serum (FBS; HyClone, Logan, UT)

at 10% (v/v), 4 mM glutamax (Invitrogen, Carlsbad, CA), NaHCO₃ (Mediatech) without antibiotics with equal number of *S. enterica* mutant and wild-type cells followed by assay of adhesion and invasion, done in triplicate, as described by Portnoy *et al.* (1988) did not reveal any difference between the competitive indexes (C.I) ($p > 0.05$) (Table 2). Since it is possible that in the competitive assay, mutant cells might benefit from the activity of simultaneously present wild-type cells, assays with RAW cells infected with the individual strains were also carried out. The results of these assays also demonstrated no difference in adhesion, invasion and intracellular persistence between wild-type and mutant strains (Table 3). Thus it appears that the role of AslA in persistence of *Salmonella typhimurium* in animals as suggested by earlier experiments is not at the level of cell adhesion, invasion or intracellular growth.

Sulfatases, a highly conserved class of enzymes, hydrolyze a wide variety of sulfate esters and sulfamates and play a role in sulfur scavenging, mucin degradation and osmoprotection (Hansen et al 2004); however, the role played by the acid sulfatase in *Salmonella enterica* in metabolism or pathogenicity and the in-vivo substrate(s) for the *aslA*-encoded sulfatases remains unknown. It is interesting that the enzyme is able to utilize two quite distinct synthetic substrates, 5-bromo-4-chloro-3-indolyl sulfate and 4-nitrophenyl sulfate, but not sugar sulfates. It is also interesting to note that apparently all sequenced *Salmonella* isolates encode AslA enzymes with little differences in their amino acid sequences, but that the only other predicted AslA-like sequences found so far are present only in *Klebsiella sp.* and in *Enterobacter massiliensis* (70 and 67% sequence identity with *Salmonella* AslA sequences, respectively). Why *Salmonella* maintains *aslA* in its genome and why similar enzymes are rare or not existent in other enteric bacteria is an interesting evolutionary question.

Conflict of Interest

The authors do not have any conflicts of interest.

Acknowledgements

The authors thank Joe Fox, William Lambert and PapaNii Asare-Okai from the Department of Chemistry and Biochemistry at the University of Delaware for synthesizing and purifying tyrosine sulfate. The study was supported by Hatch funds.

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Figure Caption

Fig. 1: Growth in the presence of potential sulfur sources as percentage of corresponding growth in the presence of 1 mM K₂SO₄. Percentage was calculated as follows: $(\text{O.D}_{600} \text{ in presence of sulfur source} - \text{O.D}_{600} \text{ in absence of sulfur source}) / (\text{O.D}_{600} \text{ in presence of 1mM K}_2\text{SO}_4 - \text{O.D}_{600} \text{ in absence of sulfur source}) * 100$. Except for 4-nitrophenyl sulfate ($p=0.015$ and 0.026 (Tuckey's test) for *S. e. Enteritis* NaIR and *Enteritidis* NaIR Δ aslA and *S. e. Kentucky 3795* and *Kentucky 3795*, respectively), no differences in growth between wild type and mutant strains was observed. Data are based on at least three independent assays.

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Table 1. Sulfatase activity as determined by p-nitrophenyl sulfate assay of cultures grown in minimal MES medium at different pHs for 24 h. The values are the average of three independent assays.

Sulfatase activity is expressed as Miller units where $1 \text{ unit} = 1000 * A_{420} - (1.75 * A_{550}) / (t * V * A_{600})$ where t =time of reaction and V =volume of culture assayed.

pH	<i>S. e. Enteritidis</i> NaI ^R	<i>S. e. Enteritidis</i> NaI ^R $\Delta as/A$	<i>S. e. Kentucky</i> 3795	<i>S. e. Kentucky</i> 3795 $\Delta as/A$
3.75	37.21± 8.88	0.00±0.01	60.85±30.95	2.80±4.81
4.0	62.10±19.81	0.00±0.01	44.27±31.08	-2.80±4.81
4.25	71.64±21.79	0.083±1.45	86.20±53.06	-1.60±2.69
4.5	106.40±10.99	1.10±1.93	65.39±47.71	0.14±0.24
4.75	130.01±33.71	0.55±0.97	65.25±42.34	-1.58±2.74
5.0	19.89±23.53	0.55±0.97	24.74±32.82	3.13±5.41
5.25	3.19±8.07	1.58±1.41	4.10±5.58	0.03±1.59
5.5	1.20±1.33	0.77±2.83	0.88±0.80	0.18±0.70
5.25	1.75±2.69	0.88±0.54	1.40±0.86	1.20±1.25
6.0	1.71±1.38	1.39±0.98	0.80±0.28	0.42±0.32
6.25	1.65±1.56	2.06±2.32	1.00±1.27	0.54±3.84
6.5	1.80±3.52	1.79±2.41	0.50±0.87	0.98±0.82

Table 2: Competitive Index values of *S. e.* wild-type and $\Delta aslA$ mutant strains during adhesion, invasion and persistence in RAW 264.7 cells

Strains	Adhesion	Competitive index ^a	
		Invasion (1 h post addition of bacteria)	Persistence (24 h post addition of bacteria)
<i>S. e.</i> Enteritidis Nal ^R / <i>S. e.</i> Enteritidis Nal ^R $\Delta aslA$	0.49 (0.26)	0.33 (0.13)	0.27 (0.47)
<i>S. e.</i> Kentucky 3795/ <i>S. e.</i> Kentucky 3795 $\Delta aslA$	0.51 (0.41)	0.25 (0.31)	0.19 (0.83)

^a Competitive index values were calculated as the output ratio of the mutant to wild type bacteria divided by input ratio of mutant to wild type bacteria. The values in parentheses represent p values.

Table3: Adhesion, invasion and persistence of *S. e. Enteritidis* Nal^R and *S. e. Kentucky* 3795 wild-type and $\Delta as/A$ cells added to RAW 264.7 cells

Strains	Inoculum added to RAW cell culture	<i>S. e.</i> cells attached to RAW cells after 1 h	<i>S. e.</i> cells internalized after 2 h	<i>S. e.</i> cells persisting in RAW cells 24 h after inoculation
<i>S. e. Enteritidis</i> Nal ^R	8.14±0.43 ^a	6.63±0.57	5.52±0.11	6.21±1.59
<i>S. e. Enteritidis</i> Nal ^R $\Delta as/A$	8.20±0.24	6.70±0.21	5.50±0.00	5.97±1.96
<i>S. e. Kentucky</i> 3795	8.07±0.53	6.54±0.19	5.71±0.40	5.87±1.39
<i>S. e. Kentucky</i> 3795 $\Delta as/A$	7.91±0.46	6.21±0.60	4.97±0.48	5.25±2.12

^a Mean log₁₀cfu/well ± standard deviation of three separate trials

Fig. 1: Growth in the presence of potential sulfur sources as percentage of corresponding growth in the presence of 1 mM K₂SO₄. Percentage was calculated as follows: (O.D₆₀₀ in presence of sulfur source – O.D₆₀₀ in absence of sulfur source)/O.D₆₀₀ in presence of 1mM K₂SO₄ – O.D₆₀₀ in absence of sulfur source)*100. Except for 4-nitrophenyl sulfate (p=0.015 and 0.026 (Tuckey’s test) for *S. e. Enteritis* NaIR and *Enteritidis* NaIR ΔasIA and *S. e. Kentucky* 3795 and *Kentucky* 3795, respectively), no differences in growth between wild type and mutant strains was observed. Data are based on at least three independent assays.

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