Characterization of an Acid-Inducible Sulfatase in \textit{Salmonella enterica} Serovar Typhimurium

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Sulfatases of enteric bacteria can provide access to heavily sulfated mucosal glycans. In this study, we show that \textit{aslA} (STM0084) of \textit{Salmonella enterica} serovar Typhimurium LT2 encodes a sulfatase that requires mildly acidic pH for its expression and activity. \textit{AslA} is not regulated by sulfur compounds or tyramine but requires the EnvZ-OmpR and PhoPQ regulatory systems, which play an important role in pathogenesis.

\textit{Salmonella} is a food-borne pathogen that affects about 94 million people worldwide each year (1). The first step in successful colonization of host intestine is to compete with the commensal bacteria for acquiring the nutrients required for growth (2). Of the various sources of nutrients available to the gut microbes, intestinal mucins are an important source of carbohydrates (2, 3). The ability to forage for mucin glycans has been shown to be important for the competition and survival of commensal \textit{Bacteroidetes} (4, 5) and for pathogenic \textit{Escherichia coli} \textit{O157:H7} (6).

Similar to the human commensal \textit{Bacteroidetes}, the genome sequences of enterobacterial human pathogens, including \textit{Salmonella}, contain many annotated sulfatase genes, indicating that sulfatases might be important for host colonization and pathogenicity by these bacteria. Very little, however, is known about the genetic and regulatory mechanisms or the physiological roles of sulfatases in enterobacteria. Early studies characterized sulfatase activity in \textit{Klebsiella aerogenes} (11, 12), \textit{Proteus rettgeri} (13), \textit{Serratia marcescens} (14), and \textit{Salmonella enterica} serovar Typhimurium (15, 16). Sulfatase activity in enteric bacteria is regulated not only by sulfur compounds but also by monoamines such as tyramine (14, 16) through a transcriptional regulator, MoaR, which also regulates enzymes for monoamine degradation (17). The details of the mechanisms by which MoaR regulates sulfatases and the physiological link to monoamine oxidation is, however, obscure.

In a systematic effort to characterize the sulfatases of \textit{Salmonella}, we evaluated various conditions for the expression of sulfatase activity using the chromogenic substrate 5-bromo-4-chloro-3-indolylsulfate (X-sulfate). Growth of \textit{S. Typhimurium} LT2 in MOPS (morpholinepropanesulfonic acid) minimal media (18) adjusted at pH 5.5, but not at pH 7.0, led to the formation of blue coloring (X-sulfate hydrolysis), indicating the presence of sulfatase activity at pH 5.5 (Fig. 1A). Consistent with earlier studies, no sulfatase activity was detected at either pH in \textit{E. coli} (Fig. 1A). To further determine whether the bacterial growth at pH 5.5 led to the induction of sulfatase or to the activation of existing sulfatase, S. Typhimurium was grown in MOPS at pH 5.5 or pH 7.0, harvested by centrifugation, and used for enzyme assays. Sulfatase activity was determined by quantifying the release of \textit{p}-nitrophenol from \textit{p}-nitrophenyl sulfate using whole cells permeabilized with chloroform in MOPS buffer at either pH 5.5 or pH 7.0 as described previously (11). Sulfatase activity was detected only in the cells grown at pH 5.5 and assayed at pH 5.5 (Fig. 1B). This indicates that the sulfatase requires pH 5.5 for its expression and for its activity.

To determine the gene encoding the acid-induced sulfatase, mutants lacking putative sulfatases genes (STM0032, STM0038, and STM0084) were constructed using P22 phage transduction (19). The mutants were grown on MOPS pH 5.5 and assayed for sulfatase activity. Of the three mutants, the acid induction of sulfatase activity was abolished in the strain lacking STM0084 (Table 2). STM0084 is annotated as \textit{aslA} and encodes a putative arylsulfatase (20). Analysis of the genomic organization of STM0084 revealed that it is not linked to a sulfatase modifying
transduction from the corresponding mutants of regulated by glucose, sulfate, and tyramine. The results are the means of at least 3 independent experiments.

Induction of sulfatase activity in mild acidic conditions suggested that it might be regulated by acid response regulatory proteins. The acid response in S. Typhimurium is regulated by different proteins, including CadC, AdiY, PhoPQ, OmpR, and RpoS. To determine if any of these proteins regulate sulfatase, mutants lacking these regulatory proteins were generated by P22 transduction from the corresponding mutants of S. Typhimurium and is likely to be a single-gene operon. Further phylogenetic analysis revealed that aslA is part of a small genomic island (STM0081 to STM0084) that is present in 50 other serovars and subspecies of S. enterica as well in Salmonella bongori. This genomic island is, however, absent in the sequenced Dublin strains (P. Desai and M. McClelland, unpublished observations).

Differential regulation of outer membrane porins in these mutants is not likely due to differential regulation of outer membrane porins in these mutants compared to the wild type. To study the combinatorial effect of OmpR and PhoP, mutants lacking both these proteins were generated as described previously (19). The ompR phoP double mutant showed activity similar to the ompR mutant (Fig. 2A). Sulfatase activity was restored to the wild-type level in the ompR mutant by complementation with phoP on a pBAD18 plasmid under the control of arabinose-induced promoter (Invitrogen Life Technologies) (Fig. 2B). However, there was significantly less sulfatase activity when OmpR was overexpressed in the envZ, phoP, or phoQ mutants (Fig. 2B). No activity was observed in the ompR mutant containing only the vector (Fig. 2B, ΔompR (VC)). These results indicate that aslA expression is likely regulated by EnvZ-OmpR and require PhoPQ for maximal expression. It is, however, also possible that these regulatory proteins affect the expression of proteins involved in the maturation of AslA, as sulfatases require posttranslational modification for their activity (10).

To differentiate between these possibilities, full-length aslA was amplified by PCR using gene-specific primers and cloned into plasmid pBAD18 under the control of an arabinose-induced promoter and used for complementation. The sulfatase activity was completely restored by the plasmid containing aslA in the aslA, envZ, ompR, phoP, and phoQ mutants when the cells were grown in the presence of arabinose. As a control, no sulfatase activity was observed in cells containing only the vector (Table 2). These results indicate that AslA is posttranslationally modified into an active form in the envZ-ompR and phoPQ mutants, and these regulatory proteins are likely involved in the expression of aslA and do not regulate sulfatase-modifying enzymes.

The role of OmpR in regulating aslA expression in acidic conditions was further explored using reverse transcription-PCR (RT-PCR). Total RNA was extracted from wild-type and ompR mutant strains using TRIzol (Sigma), reverse transcribed, and am-

**TABLE 1 Acid-induced sulfatase activity of S. Typhimurium is not regulated by glucose, sulfate, and tyramine**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sulfatase activity (Miller units)</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>557 ± 45</td>
</tr>
<tr>
<td>ΔaslA (pBAD-aslA)</td>
<td>17 ± 4</td>
</tr>
<tr>
<td>ΔenvZ (pBAD-aslA)</td>
<td>744 ± 55</td>
</tr>
<tr>
<td>ΔompR (pBAD-aslA)</td>
<td>1046 ± 99</td>
</tr>
<tr>
<td>ΔphoP (pBAD-aslA)</td>
<td>770 ± 23</td>
</tr>
<tr>
<td>ΔaslA (pBAD)</td>
<td>975 ± 93</td>
</tr>
<tr>
<td>ΔoslA (pBAD)</td>
<td>10 ± 5</td>
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* Overexpression of aslA leads to active sulfatase in the envZ-ompR and phoP mutants, indicating that the posttranslational maturation of AslA is independent of these two regulatory systems. Sulfatase was assayed at pH 5.5 by quantifying the release of p-nitrophenol from p-nitrophenyl sulfate using whole cells permeabilized with chloroform and expressed as Miller units. The results are the means ± standard deviations of at least 3 independent experiments.
plified using *aslA*-specific primers. Expression of *recA* was determined as a control using *recA*-specific primers. In accordance with the results showing sulfatase activity only at pH 5.5 (Fig. 1), *aslA* expression was not detected when the cells were grown at pH 7.0 (Fig. 3, lane 1) but was detected at pH 5.5 (Fig. 3, lane 2). The expression of *aslA* was not detected in the *ompR* mutant (Fig. 3, lane 3). To determine if OmpR regulates *aslA* directly or indirectly, wild-type *S. Typhimurium* was grown at pH 7.0 and shifted to pH 5.5 with or without chloramphenicol (inhibits protein synthesis) and incubated for an additional 6 h. The cells were harvested, and expression of *aslA* and *recA* (as a control) was monitored by RT-PCR. Addition of chloramphenicol abolished the expression of *aslA* but had no effect on the expression of *recA* (Fig. 3, lane 4), indicating that chloramphenicol did not affect the general transcriptional abilities of the cells in the assay. These results suggest that OmpR likely regulates *aslA* via the synthesis of additional transcriptional factor(s) that need to be further characterized.

A recent study has suggested that *aslA* contains the twin-arginine translocase (TAT) signal and is likely to be secreted by the TAT pathway of *S. Typhimurium* (20). To further determine the subcellular localization and the role of TAT in AslA secretion, we determined the sulfatase activity in the extracellular, periplasmic, and cell fractions in the wild-type strain using the PeriPreps periplastaing kit (Epicenter Biotechnology, Madison, WI). Sulfatase activity was not detected in the extracellular or periplasmic fraction but was localized in the spheroplastic fraction, indicating that AslA is either localized to the cytoplasmic membrane or is intracellular (data not shown). To further differentiate these possibilities, sulfatase activity was assayed with and without chloroform (to permeabilize the membrane) in the wild-type and TatC mutant strains. Addition of chloroform did not have any significant effect on activity in the wild-type strain. In the TatC

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**FIG 2** AslA is regulated by the PhoPQ and EnvZ-OmpR regulatory systems. (A) The sulfatase activity was reduced 2-fold in mutants lacking *phoPQ* and was reduced 50-fold in mutants lacking *envZ-ompR* compared to the wild type. Mutants affected in *cadC*, *adiY*, and *tpoS* had activity similar to the wild type. (B) Complementation by plasmid-based *ompR* resulted in restoration of sulfatase activity in the *ompR* mutant but was significantly less in the *envZ*, *phoP*, and *phoQ* mutants. *, *P* < 0.01; **, *P* < 0.001. Sulfatase was assayed by quantifying the release of *p*-nitrophenol from *p*-nitrophenyl sulfate using whole cells permeabilized with chloroform and expressed as Miller units (27). The results are the means ± standard deviations of at least 3 independent experiments. No activity was detected in the *ompR* mutant containing only the plasmid vector (VC).

**FIG 3** Analysis of *aslA* expression by RT-PCR. *aslA* was not expressed in cells grown at pH 7.0 (lane 1). The expression was induced when cells were shifted to pH 5.5 (lane 2). No expression was detected in the *ompR* mutant (lane 3). Addition of chloramphenicol after shifting the wild-type cells to pH 5.5 led to inhibition of *aslA* expression (lane 4). Expression of *recA* was used as a control.
TABLE 3 Effect of chloroform addition on acid-induced sulfatase activity in the wild-type and ΔtatC mutant strains of S. Typhimurium

<table>
<thead>
<tr>
<th>Strain</th>
<th>Chloroform</th>
<th>Sulfatase activity (Miller units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>–</td>
<td>557 ± 45</td>
</tr>
<tr>
<td>Wild type +</td>
<td>+</td>
<td>455 ± 90</td>
</tr>
<tr>
<td>ΔtatC</td>
<td>–</td>
<td>15 ± 5</td>
</tr>
<tr>
<td>ΔtatC +</td>
<td>+</td>
<td>620 ± 55</td>
</tr>
</tbody>
</table>

*Sulfatase was assayed at pH 5.5 by quantifying the release of p-nitrophenol from p-nitrophenyl sulfate using whole cells permeabilized with chloroform and expressed as Miller units. The results are the means ± standard deviations of at least 3 independent experiments.

mutant strain, however, activity was detected only in the presence of chloroform (Table 3). Taken together, these results suggest that AslA is likely to be secreted by the TAT system to the cytoplasmic membrane.

In S. Typhimurium, envZ-ompR and phoPQ are major regulators of virulence (22, 23). The PhoQ sensor protein is responsive to low magnesium concentrations and acidic pH, and the PhoP response regulator affects the expression of many genes that are important for adaptation to the mildly acidic conditions (pH 5.0) of the intraphagosomal environment (23–25). Similar to the phoPQ regulatory system, EnvZ-OmpR activates the expression of phoPQ of the intraphagosomal environment (23–25). Similar to the phoPQ regulatory system, EnvZ-OmpR activates the expression of genes in the pathogenicity island 2 (SPI-2), which is crucial for Salmonella infection of macrophages (22, 26). Additionally, the TAT protein secretion system was recently shown to be important for S. Typhimurium virulence (20). Taken together, these results indicate that the acid induction of AslA by OmpR and PhoP and its secretion by the TAT system likely have a physiological role in S. Typhimurium survival and pathogenesis. The role of AslA in S. Typhimurium virulence will be the subject of future study.

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