**Bacillus anthracis** anthrolysin O and three phospholipases C are functionally redundant in a murine model of inhalation anthrax

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**Keywords**

*Bacillus anthracis*; phospholipase C; anthrolysin O; anthrax; virulence.

**Abstract**

Although traditionally considered to be an extracellular pathogen, *Bacillus anthracis* has a brief intracellular step to initiate anthrax. At the onset of infection, *B. anthracis* must withstand the bactericidal activities of the macrophage. Recently, three phospholipases C (PLCs) were shown to contribute to macrophage-associated growth of *B. anthracis* by presumably aiding in the escape of the bacterium from phagocytic vacuoles following phagocytosis. However, in the absence of all three PLCs, vegetative bacilli were still observed growing in association with the macrophage, albeit to a lesser extent, implicating that additional factors are involved in this process. In this study, the contributions of the previously identified cholesterol-dependent cytolysin anthrolysin O (ALO) to *B. anthracis* pathogenesis were investigated following challenges of bone marrow-derived macrophages and intratracheal inoculations of mice. Disruption of ALO alone yielded no differences in virulence in mice. However, combinatorial deletions of ALO with the three PLCs resulted in attenuation in both tissue culture and murine challenges, suggesting that these toxins may have overlapping roles in anthrax pathogenesis.

**Introduction**

While each branch of the immune system works in concert to defend its host, often the effectiveness of early, innate defenses, such as those provided by phagocytes, determines the outcome of an infection. In anthrax, the phagocytes proximal to the portal of entry of the infectious *Bacillus anthracis* endospores into the body are responsible for the onset of infection. Several models of anthrax pathogenesis suggest that, within these host cells endospores germinate, outgrow into vegetative bacilli, and are ultimately released into the blood and lymph where they express exotoxins and replicate to very high numbers (Ross, 1957; Guidi-Rontani et al., 1999; Dixon et al., 2000).

Defining and understanding the full repertoire of virulence factors of *B. anthracis* remains an important goal for discerning key intervention points of the anthrax infectious cycle. In addition to the plasmid-encoded capsule and binary toxin complexes, factors encoded on the chromosome are now beginning to be investigated as potential virulence factors. These include homologues of membrane-active toxins associated with virulence in other Gram-positive bacterial infections, namely three phospholipases C (PLCs) and a pore-forming cytolysin termed anthrolysin O, (ALO) (Klichko et al., 2003; Pomerantsev et al., 2003; Shannon et al., 2003; Wei et al., 2005; Heffernan et al., 2006).

PLCs cleave polar head groups from phospholipids, leading to membrane disruption and generation of lipid secondary messengers (Titball, 1998). They also play prominent roles in the pathogenesis of other Gram-positive bacterial species (Goldfine et al., 1998; Flores-Diaz & Alaped-Giron, 2003). Phosphatidylycerol (PC), phosphatidylinositol-specific phospholipases C (PI-PLC) and sphingomyelinase activities, encoded for by *plcB*, *plcA*, and *smcA* respectively in *B. anthracis*, are well described in the Listerial system for their biochemical and membrane damaging properties as well as their interactions with the pore-forming cytolysin, listeriolysin O (LLO) (Smith et al., 1995; Gonzalez-Zorn et al., 1999). Recently, the three PLCs expressed by *B. anthracis* were shown to contribute to anthrax pathogenesis in a murine model of infection (Heffernan et al., 2006). Defined disruptions of any one or two of the PLCs resulted in little to no change in virulence as compared with the isogenic parental, but the loss of all three PLCs caused roughly a ninefold increase in LD₅₀ (Heffernan et al., 2006). We hypothesized that this
moderate reduction in virulence measured for the triple PLC null strain could potentially be due to further redundancy/synergy between the PLCs and ALO.

Initial studies have identified ALO as a member of the cholesterol-dependent cytolsin (CDC) family of pore-forming toxins that are produced by more than 20 Gram-positive species, including those from the genera Listeria, Clostridium, Streptococcus, Arcanobacterium and Bacillus (Shannon et al., 2003; Tweten, 2005). CDCs are secreted as soluble monomers that localize exclusively to cholesterol containing membranes which upon binding and insertion form a large, oligomeric pore (Tweten, 2005). The formation of large pores in cholesterol-containing membranes is responsible for the cytolytic properties of CDCs.

Although ALO was shown to have cytolytic activities and was capable of functionally complementing the escape phenotype in an Listeria monocytogenes strain lacking LLO, a formal role for ALO in anthrax pathogenesis has not yet been elucidated (Shannon et al., 2003; Wei et al., 2005). Since the infectious lifecycle of B. anthracis has been elucidated (Shannon et al., 2003; Tweten, 2005), we hypothesized that ALO, in a manner similar to LLO, may work in conjunction with the PLCs during the early intracellular stages of anthrax. Here we describe the further characterization of ALO and investigate its contributions to B. anthracis pathogenesis in a murine model of inhalation anthrax and during challenges of bone-marrow derived macrophages (BMM), alone and in conjunction with the three PLCs.

### Materials and methods

#### Growth conditions and strain construction

Bacterial strains, plasmids, and phages relevant to this study are listed in Table 1. Bacillus anthracis strains were cultured for vegetative growth in brain–heart infusion (BHI, Difco) medium while Luria–Bertani (LB) medium was used to cultivate Bacillus subtilis and Escherichia coli strains. Media was supplemented with antibiotics to maintain selection at

<table>
<thead>
<tr>
<th>Strains/plasmids/phages</th>
<th>Relevant genotype</th>
<th>Sources/references</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. anthracis Sterne 7702</td>
<td>pXO1°, pXO2°</td>
<td>Pezard et al. (1993)</td>
</tr>
<tr>
<td>BDT101</td>
<td>7702, ΔaloA::km’</td>
<td>This study</td>
</tr>
<tr>
<td>BJH252</td>
<td>7702, ΔaloA::km’ ΔiplB::sp’</td>
<td>This study</td>
</tr>
<tr>
<td>BJH253</td>
<td>7702, ΔaloA::km’ ΔsmcA::sp’</td>
<td>This study</td>
</tr>
<tr>
<td>BJH254</td>
<td>7702, ΔaloA::km’ ΔiplA::em’</td>
<td>This study</td>
</tr>
<tr>
<td>BJH255</td>
<td>7702, ΔaloA::km’ ΔiplBsmcA::sp’</td>
<td>This study</td>
</tr>
<tr>
<td>BJH256</td>
<td>7702, ΔaloA::km’ ΔiplB::sp’ ΔiplA::em’</td>
<td>This study</td>
</tr>
<tr>
<td>BJH257</td>
<td>7702, ΔaloA::km’ ΔsmcA::sp’ ΔiplC::em’</td>
<td>This study</td>
</tr>
<tr>
<td>BJH258</td>
<td>7702, ΔaloA::km’ ΔiplBsmcA::sp’ ΔiplC::em’</td>
<td>This study</td>
</tr>
<tr>
<td>B. subtilis KY42</td>
<td>Contains a silent transposon Tn917 insertion in which the ermC region was replaced with a chloramphenicol resistance gene</td>
<td>Freitag et al. (1992)</td>
</tr>
<tr>
<td>E. coli DH5x</td>
<td>F- ΔlacZYA-argF-U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 thi-1 gyrA96 relA1-</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>One shot TOP10</td>
<td>F- mcrA Δmrr-hsdRMS-mcrBC Δ80lacZ ΔM15 ΔlacX74 recA1 arad139A (araIeu)7697 galU galK rpsL (Strr) endA1 nupG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>One shot INV110</td>
<td>F’ [traD36 proAB lacI (lacZΔM15) rpsL (Strr) thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44X(lac-proAB)Δ(mcrC-mrr) 102::Tn10 (Tetr)]</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Plasmids pUC19</td>
<td>pBRR22 derivative lacZα ap’</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>pDG783</td>
<td>pSB118::km’</td>
<td>Guerout-Fleury et al. (1995)</td>
</tr>
<tr>
<td>pCR-XL-TOPO</td>
<td>P lacZα ccdB km’ pUC19</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pKSV7</td>
<td>pUC19 (pE194kan’ ap’ cmr’</td>
<td>Smith &amp; Youngman (1992)</td>
</tr>
<tr>
<td>pH13</td>
<td>pUC19 (pTA1060cmr’ emr’</td>
<td>Haima et al. (1987)</td>
</tr>
<tr>
<td>pBTO01</td>
<td>pKSV7 (ΔaloA::km’)</td>
<td>This study</td>
</tr>
<tr>
<td>pBTO02</td>
<td>pAG58::aloA</td>
<td>This study</td>
</tr>
<tr>
<td>pBH095</td>
<td>pH13::iplBsmcAaplA</td>
<td>Heffernan et al. (2006)</td>
</tr>
<tr>
<td>pBH096</td>
<td>pH13::aloA</td>
<td>This study</td>
</tr>
<tr>
<td>Bacteriophage CP-51</td>
<td>Generalized transducing phage</td>
<td>Thorne (1968)</td>
</tr>
</tbody>
</table>

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the following concentrations: ampicillin (100 μg mL⁻¹), chloramphenicol (10 μg mL⁻¹), erythromycin (1 μg mL⁻¹), kanamycin (50 μg mL⁻¹), phleomycin (1 μg mL⁻¹), and spectinomycin (100 μg mL⁻¹). Bacillus anthracis was cultured in CCY medium for sporulation (Stewart et al., 1981). Endospores were prepared as previously described (Dixon et al., 2000). Plasmid constructs were made in E. coli backgrounds and passed through methylation deficient E. coli strains before being introduced into either B. anthracis or B. subtilis. Primers were designed using the genome sequence of the B. anthracis Ames strain (GenBank accession number AE016879). PCR amplification was performed using the genomic template isolated from Sterne 7702, and the nucleotide sequence of amplified fragments was confirmed by sequence analysis.

The B. anthracis ALO-null strain (ΔaloA) was constructed in the Sterne 7702 background using an allelic replacement technique as described previously (Cendrowski et al., 2004). Briefly, the aloA gene was amplified using the primers 5'-GGATCCGTATCATCTTCAGCATAATT-3' and 5'-AACTGCAGAACCAATGCATTGGGAGAGA-3'. A 2.8-kb fragment was cloned first into pCR-XL-TOPO (Invitrogen) according to the manufacturer's specifications and then moved into pKSV7. The pKSV7:ΔaloA construct was generated by ligating an inverse-PCR amplified product of pKSV7:aloA (5'-GGGAGCGCTACATGTTGAGGAGGAGGAGGAGGAC-3' and 5'-CGGGATCCATATCTCTACTATCATACGTGCAATCTGATGATT-3') and a PCR fragment of pDG783 containing the kanamycin resistance cassette (5'-GGGCGCGCCCAATACAGATTATATCGAG-3'). The resulting plasmid was mobilized into B. subtilis strain 168, using a conjugation protocol as described by the manufacturer.

Electron microscopy

Infections of RAW264.7 cells with B. subtilis strains were done in a manner identical to the above procedure except that challenges were performed in 150 cm² tissue culture flasks. Cells were collected at 4 h postinfection by gentle scraping and subsequent centrifugation at 1000 g for 5 min. Cells were fixed in 2.5% glutaraldehyde in 0.1 M Sorensen's buffer (Bozzola & Russell, 1992) and postfixed in 1% osmium tetroxide. After en bloc staining with aqueous 2% uranyl acetate, cells were dehydrated in a graded series of ethanol washes. Cell pellets were embedded in Spurr's resin (Bozzola & Russell, 1992) and polymerized. Ultrathin sections were stained with uranyl acetate and lead citrate and then examined on a Philips CM100 electron microscope.

Infections of BMM

BMM obtained from 6–8-week-old female Balb/c mice (Jackson Laboratories) were seeded in 24-well tissue culture plates at a concentration of 1 x 10⁵ cells per well and challenged with B. anthracis endospores at a multiplicity of infection of 10 endospores per macrophage. Infections were initiated with a brief 10-min spin at 100 g to promote contact between the endospores and the macrophages. Infections were allowed to proceed for 20 min to allow for...
uptake of endospores into the cells. Monolayers were washed three times with Dulbecco’s modified Eagle medium (DMEM), and the media was replaced with DMEM, 10% FBS supplemented with a gentamicin/germinant solution to allow for germination and reduction of extracellular bacteria as described previously (Heffernan et al., 2006). After 30 min, the monolayers were washed an additional three times with DMEM and the media was replaced with fresh DMEM 10% FBS. Supernatants were aspirated at the indicated time points, and the macrophages were scraped in 0.2% saponin. CFUs were enumerated by plating serial dilutions on BHI plates. Changes in cell-associated growth were normalized to viable counts at the 0 h time point. Infections were done identically as described above for the evaluation of cytotoxicity except at 8 h postgentamicin treatment, supernatants were collected and macrophage cytotoxicity was measured by release of lactate dehydrogenase (LDH) with the CytoTox-ONE Homogeneous Membrane Integrity Assay (Promega). Percent cytotoxicity was calculated as $100 \times \frac{(\text{experimental LDH release}) - (\text{spontaneous LDH release})}{(\text{maximum LDH release}) - (\text{spontaneous LDH release})}$.

**Mice infections**

Six-to-eight-week-old female DBA/2J mice (Jackson Laboratories) were utilized for intratracheal inoculations. Mice were anesthetized by intraperitoneal injection of Ketamine (120 mg kg$^{-1}$) and Xylazine (5 mg kg$^{-1}$). Mice were restrained on a surgical board and a small incision was made through the skin above the trachea. After the underlying tissue was separated, a 30-gauge needle was inserted into the trachea to deliver a 30 µL dose of *B. anthracis* endospores directly into the lungs. The following doses were administered for each of the ALO/PLC null strains: 10$^2$, 10$^3$, 10$^4$, 10$^5$, and 10$^6$ endospores, with a total of eight mice per dose used. Aliquots of the inoculums were plated to monitor the number of CFU delivered. Mice were observed for a period of 2 weeks with a majority of mortalities occurring over the first few days. Following mortality, *B. anthracis* strains were recovered from the spleen, blood and/or lungs and confirmed. Methods described by Reed & Muench (1938) were used to estimate the LD$_{50}$. MTD was calculated by averaging the time of death for all individuals who died after receiving 10$^5$ endospores.

**Results and discussion**

**Ectopic expression of aloA in B. subtilis confers phagosomal escape to the recombinant bacterium**

Previously, it was shown that ectopic expression of CDCs in *B. subtilis* enables this normally escaping incompetent bacterium to gain access to the host cell cytosol during tissue culture infections. Recombinant *B. subtilis* strains expressing LLO and perfringolysin O (PFO) were able to lyse phagocytic vacuoles and grow directly in the cytosol of cultured macrophages (Portnoy et al., 1992). However, not all CDCs are able to impart this ability to *B. subtilis* as streptolysin O from *Streptococcus pyogenes* was shown to be ineffective at mediating the growth of *B. subtilis* within macrophages (Portnoy et al., 1992). In order to determine whether ALO can facilitate vacuolar escape and allow *B. subtilis* to grow within the macrophage cytosol, a plasmid construct was made in which aloA was put under the control of an IPTG-inducible P$_{spac}$ promoter. The resultant plasmid, pAG58::aloA, was integrated into the *B. subtilis* genome to create the *Bs-aloA* strain, which is able to secrete ALO in the presence of 10 mM IPTG.

No growth was observed following challenges of macrophages with the *B. subtilis* KY42 strain as expected (Fig. 1). The *Bs-aloA* strain also did not survive macrophage challenges when infections were done in the absence of IPTG (Fig. 1). Expression of ALO, upon the addition of IPTG to the medium, also resulted in an equivalent decrease in CFU over the first 2 h of the infection (Fig. 1). The rate of death for the *Bs-aloA* strain, however, was slowed over the next 6 h of the infection and eventually leveled off by 12 h postchallenge when IPTG was present (Fig. 1). These results indicate that expression of ALO allows *B. subtilis* to resist the bactericidal effects of the macrophage, but it does not confer the ability of the bacterium to grow within the phagocytic...
cell. The lack of growth may be a consequence of weak escape by the recombinant bacterium into the cytosol resulting from the inefficient capabilities of ALO to mediate escape alone. Meager pore formation may still allow release of vacuolar contents and increase the phagosomal pH, thus aiding in survival. Alternatively, the absence of growth could be reflective of the inability of the *B. subtilis* KY42 strain to grow within macrophages. Although it has previously been reported that *B. subtilis* expressing LLO is capable of multiplying in host cells after escape from vacuoles, other studies have indicated that *B. subtilis* is unable to grow within the cytosol following microinjection into the host cell (Bielecki et al., 1990; Goetz et al., 2001). For this reason, transmission electron microscopy was utilized to determine whether the ΔaloA and ΔaloAΔpLCΔbsmcΔaplCΔA strains compared with the Sterne 7702 strain were significant (P < 0.02 by unpaired two tailed t test). The differences in the fold increase in growth at 8 h post gentamicin treatment between the ΔaloA strain compared with the ΔaloAΔpLCΔbsmcΔaplCΔA strain were significant (P < 0.05 by unpaired two tailed t test).

**Cooperation between ALO and the PLCs mediates macrophage associated growth and survival**

Previously, three PLCs were shown to contribute to the growth of *B. anthracis* in association with macrophages (Heffernan et al., 2006). However, even in the absence of all three PLCs, vegetative bacilli were still observed growing in association with the macrophage, albeit to a lesser extent, suggesting that additional factors are involved in BMM associated growth, potentially including the recently identified cytolysin, ALO. To determine whether the combined actions of ALO and the three PLCs aid *B. anthracis* during macrophage challenges, BMM were infected with ALO/PLC null strains and scored for their ability to grow and cause macrophage cell death.

The deletion of *aloA* alone resulted in a diminished capacity of *B. anthracis* to grow in association with the macrophage and resulted in approximately a 50% reduction...
in cytotoxicity by scoring for the release of the cytoplasmic LDH. Experiments were done in triplicate, and the average of three representative experiments are depicted here with SD. The differences in LDH release between the ΔaloA and ΔaloAplcBsmcApICa strains compared to the Sterne 7702 strain were significant (P < 0.02 by unpaired two tailed t test). The differences between the ΔaloA strain compared with the ΔaloAplcBsmcApICa strain were significant (P < 0.02 by unpaired two tailed t test).

**Combinatorial deletions of ALO with the three PLCs results in further attenuation in a murine model of inhalation anthrax**

Since ALO contributed to bacterial growth and survival during challenges of cultured BMM, a murine model was employed to determine whether ALO had a role in anthrax pathogenesis *in vivo*. Even though the ΔaloA strain displayed a defect in growth in association with macrophages, no decrease in virulence was detected for this strain in the inhalation anthrax model (Table 2). This could imply that the ΔaloA strain is able to overcome its defect in macrophage-associated growth to retain virulence equivalent to Sterne or, alternatively, that *B. anthracis* may have additional, macrophage-independent means to disseminate during anthrax infections in which ALO is expendable. In support of the latter possibility is the recent finding that endospores germinate and produce successful infections in macrophage-depleted mice (Cote et al., 2004). Thus, while macrophages might play a key role during normal infections, their presence is not absolutely required for the progression of anthrax. Dendritic cells (DC), another phagocytic cell associated with the innate immunity of the lungs, may potentially facilitate anthrax in a role analogous to the macrophage as DCs were shown to engulf and support the germination of *B. anthracis* endospores (Brittingham et al., 2005).

**Table 2.** LD50 and MTD values for mice challenged intratracheally with *Bacillus anthracis* ALO/PLC null strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>LD50 (day)</th>
<th>MTD (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterne 7702</td>
<td>8.15 × 10⁴</td>
<td>2</td>
</tr>
<tr>
<td>ΔaloA</td>
<td>1.03 × 10⁵</td>
<td>2</td>
</tr>
<tr>
<td>ΔaloAplcB</td>
<td>1.01 × 10⁴</td>
<td>2</td>
</tr>
<tr>
<td>ΔaloAasmcA</td>
<td>3.25 × 10⁵</td>
<td>2</td>
</tr>
<tr>
<td>ΔaloApICa</td>
<td>7.90 × 10⁴</td>
<td>3</td>
</tr>
<tr>
<td>ΔaloAplcBsmcA</td>
<td>3.31 × 10⁵</td>
<td>4¹</td>
</tr>
<tr>
<td>ΔaloAasmcApICa</td>
<td>3.35 × 10⁵</td>
<td>2</td>
</tr>
<tr>
<td>ΔaloApICapICa</td>
<td>4.94 × 10⁴</td>
<td>4¹</td>
</tr>
<tr>
<td>ΔaloAplcBsmcApICa (pBH095)</td>
<td>3.17 × 10⁵</td>
<td>4¹</td>
</tr>
<tr>
<td>ΔaloAplcBsmcApICa (pBH095)</td>
<td>9.91 × 10³</td>
<td>3</td>
</tr>
</tbody>
</table>

*LD50 values were calculated using the methods described by Reed & Muench (1938).

¹MTD determined for 10³ endospores dose.

²P < 0.02 compared with Sterne 7702 by log rank test.
The lack of attenuation for the ΔaloA strain could result from redundancy shared between ALO and the previously characterized PLCs. The deletion of aloA in either the ΔpclB or ΔplcA backgrounds, however, did not result in any major differences in virulence in relation to the Sterne strain (Table 2). Conversely, the combined disruption of aloA, plcB, and plcA did yield a decrease in lethality compared with the parental strain, with a sixfold increase in LD₅₀ (Table 2). The ΔaloAsmcA strain was also shown to have a higher LD₅₀ than the Sterne strain (Table 2). The fourfold increase in LD₅₀ for the ΔaloAsmCA strain suggests that ALO and sphingomyelinase activities are possibly redundant in function as disruption of either factor alone led to no changes in virulence. Further deletion of plcB or plcA in the ΔaloAsmCA strain also resulted in a fourfold increase in LD₅₀ compared with the parental Sterne, suggesting that the additional loss of PC-PLC or PI-PLC activities in these strains had no effect on virulence (Table 2). The largest attenuation was scored for the quadruple null strain, with all four membrane-active toxins disrupted. A c. 40-fold reduction in virulence compared with the Sterne parental strain was scored for the ΔaloAplcBsmcAplcA strain (Table 2). This increase in LD₅₀ was also accompanied by a statistically significant increase in the MTD of 2 days (Table 2). The attenuation in virulence observed for the ΔaloAplcBsmcAplcA strain was able to be restored to levels equivalent to the ΔaloA strain by expression of the PLC genes in trans, again demonstrating that the differences seen between these two strains was the result of the further loss of the PLCs (Table 2).

Upon analysis of these data, it becomes clear that ALO, in conjunction with the PLCs, contributes to B. anthracis pathogenesis. These observations, in addition to the previous report on the PLCs of B. anthracis, suggest that B. anthracis must express ALO with at least one functional PLC, or that sphingomyelinase activity must be present with the addition of either PI-PLC or PC-PLC activities for no attenuation in virulence to occur during intratracheal infections of mice (Table 2; Heffernan et al., 2006). The attenuation in mice may be a direct consequence of the inability of the ALO/PLC null strains to grow in association with macrophages, although these toxins may also contribute to anthrax at other points in the infection.

The cooperation that exists between the PLCs and its functional redundancy that exists between these four toxins can compensate for the deletion of any one of these factors without resulting in any attenuation in virulence in a murine model of anthrax. The redundancy between these toxins might be a fail-safe mechanism to assure the survival of B. anthracis within the host.

Acknowledgements

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Disclaimer

The experiments reported herein were conducted in compliance with the Animal Welfare Act and in accordance with the principles set forth in the ‘Guide for the Care and Use of Laboratory Animals,’ Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 1996. The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, or of the US Government.

Authors contribution

B.J.H. and B.T. contributed equally to this study.

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