**Helicobacter pylori** Outer Membrane Vesicles Protect the Pathogen From Reactive Oxygen Species of the Respiratory Burst

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Outer membrane vesicles (OMVs) play an important role in the persistence of *Helicobacter pylori* infection. *Helicobacter* OMVs carry a plethora of virulence factors, including catalase (KatA), an antioxidant enzyme that counteracts the host respiratory burst. We found KatA to be enriched and surface-associated in OMVs compared to bacterial cells. This conferred OMV-dependent KatA activity resulting in neutralization of \(\text{H}_2\text{O}_2\) and \(\text{NaClO}\), and rescue of surrounding bacteria from oxidative damage. The antioxidant activity of OMVs was abolished by deletion of KatA. In conclusion, enrichment of antioxidative KatA in OMVs is highly important for efficient immune evasion.

**Keywords:** *H. pylori*, KatA, outer membrane vesicles, oxidative burst, reactive oxygen species

**INTRODUCTION**

*Helicobacter pylori* is a Gram-negative pathogen that commonly colonizes the gastric mucosa. Infection persists for a lifetime without antibiotic treatment although the pathogen constantly experiences hostile conditions including the acidic ventricle environment and host defense (Roesler et al., 2014). In order to survive against the highly acidic gastric juice (pH 1.0–3.0), *H. pylori* uses a series of acidic acclimation systems that neutralize the surrounding acid. Other virulence mechanisms include expression of abundant molecules at the surface for attachment and manipulation of host extracellular matrix proteins and serum resistance (Parker and Keenan, 2012; Richter et al., 2016). In addition, *H. pylori* is equipped with antioxidant molecules such as catalase (KatA), catalase-like protein (KatB), alkyl hydroperoxide reductase (AhpC), and superoxide dismutase (SOD) to detoxify reactive oxygen species (ROS) released from host immune cells during the respiratory burst (Wang et al., 2006). Furthermore, *H. pylori* constitutively releases outer membrane vesicles (OMVs) from its outer membrane (OM).

Outer membrane vesicles are cargos comprising an OM lipid bilayer enveloping several virulence factors. *H. pylori* OMVs have been extensively studied with respect to composition, proteome, and virulence functions (Mullaney et al., 2009; Olofsson et al., 2010), and play multiple roles in bacterial pathogenesis including biofilm formation, cancer development, and immune evasion (Parker and Keenan, 2012). Furthermore, OMVs display immunomodulatory effects by inducing IL-8 secretion from epithelial cells, activating phagocyes, and suppressing immune cells of the adaptive immune system (Mullaney et al., 2009; Olofsson et al., 2010; Ko et al., 2016).
KatA, a 55 kDa catalase, is an essential virulence factor protecting *H. pylori* against the respiratory burst (Olofsson et al., 2010). In fact, KatA is upregulated during oxidative stress (Huang and Chiou, 2011). It is widely known that KatA detoxifies hydrogen peroxide (H$_2$O$_2$) and hypochlorite (OCl$^-$) (Benoit and Maier, 2016). Additionally, we recently reported that KatA mediates vitronectin acquisition resulting in increased serum resistance (Richter et al., 2016). Interestingly, despite the lack of a signal peptide, *Helicobacter* KatA is ubiquitous with various topology including the bacterial surface, the cytosol and periplasmic space. KatA has also recently been identified in OMVs (Wang et al., 2006; Mullaney et al., 2009). However, little is known regarding the role of KatA in OMVs since previous studies have mainly focused on KatA in the cell-associated context.

We determined the importance of OMVs to eliminate extracellular ROS-mediated killing via KatA enrichment. Our data suggest a new mechanism of OMV-mediated *H. pylori* evasion from the attack of the innate immune system.

**RESULTS**

**KatA Catalase Is Enriched in *H. pylori*-Derived OMVs**

*Helicobacter pylori* KatA has been predicted as one of the periplasmic proteins that accounts for 7.4% of the total OM proteome (Mullaney et al., 2009; Olofsson et al., 2010). Since most OM proteins are also located at the surface of vesicles (Bonnington and Kuehn, 2014), we wanted to investigate whether KatA localizes at the outer surface of *H. pylori* OMVs. As visualized by TEM, we found deposition of gold-labeled anti-KatA pAb at the surface of intact bacteria and OMVs of *H. pylori* wild type (wt) (Figure 1A). However, no KatA was detected on any samples derived from the KatA-deficient *H. pylori* ΔkatA mutant. This suggested a similar surface exposure of KatA on OMVs as seen on intact bacteria. Further enumeration of antibody deposition revealed that more KatA was detected at the "blebbing areas" of wild type bacteria as compared to the "non-blebbing areas," and this appearance was almost similar to the OMVs (Figure 1B). This observation prompted our interest to compare the amount of KatA present in the OMVs and OM of *H. pylori*. Interestingly, we observed that OMVs contained sevenfold more KatA (18.37 ± 6.24 ng/µg sample) than bacterial OM (2.42 ± 0.24 ng/µg sample) (Figure 1C and Supplementary Figure S1).

**KatA Enriched OMVs Exhibit Catalase Activity**

*Helicobacter* KatA of intact bacteria is known to actively hydrolyse H$_2$O$_2$ and detoxify ClO$^-$ (Wang et al., 2006; Benoit and Maier, 2016). Interestingly, we found that the H$_2$O$_2$ hydrolysis activity in OMVs was significantly ($p < 0.05$) higher than the *H. pylori* wt whole cell lysate (Figure 2A). We further investigated whether KatA could contribute to the antioxidiant activity of *Helicobacter* OMV. As shown in Figure 2B, OMVs isolated from the strain *H. pylori* 18943 wt exhibited a strong
H. pylori also found with OMVs isolated from another strain. KatA-deficient H. pylori (P12), whereas no activity was observed with the corresponding heat-inactivated KatA (OMV\textsuperscript{wt}) was able to neutralize H\textsubscript{2}O\textsubscript{2} and promote survival of H. pylori P12 from peroxidative killing. OMV lacking KatA (OMV\textsuperscript{katA\_lacking}) or heat-inactivated KatA (OMV\textsuperscript{katA\_lacking}) did not show any protection of whole bacteria (mean ± SD; n = 3; *p < 0.05; **p < 0.01). In (D), the bactericidal activity of OMVs was decreased by preincubation with increasing amounts of OMV\textsuperscript{wt} (equivalent to OMVs produced from 0.2 to 2 × 10\textsuperscript{8} CFU). The killing of H. pylori 18943ΔkatA by NaOCl was measured by the diameter of inhibition zone, which is the area without bacterial growth (mean ± SD; n = 3; **p < 0.01; ***p < 0.001).}

KatA-Enriched OMVs Promote H. pylori Survival Against the ROS of the Oxidative Burst

We also wanted to determine whether OMV loaded with KatA could protect bacteria from the bactericidal activity of ROS (H\textsubscript{2}O\textsubscript{2} and ClO\textsuperscript{−}) (Wang et al., 2006; Benoit and Maier, 2016). First, H. pylori ΔkatA lacking the catalase activity was exposed to H\textsubscript{2}O\textsubscript{2} that had been pre-incubated with OMVs derived from H. pylori wt (OMV\textsuperscript{wt}) or the KatA-deficient mutant (OMV\textsuperscript{ΔkatA}). Interestingly, H. pylori ΔkatA survived when OMV\textsuperscript{wt}-pre-treated H\textsubscript{2}O\textsubscript{2} was added, but was completely killed in both H\textsubscript{2}O\textsubscript{2} or H\textsubscript{2}O\textsubscript{2} preincubated with OMV\textsuperscript{ΔkatA} (Figure 2C). Since KatA activity is heat sensitive, OMV\textsuperscript{wt} was also heat-inactivated at 60°C to generate OMV\textsuperscript{wt–hia}. We found that H\textsubscript{2}O\textsubscript{2} preincubated with OMV\textsuperscript{wt–hia} remained bactericidal against the mutant H. pylori ΔkatA.

We subsequently performed a disk diffusion assay to examine the capacity of OMVs in protecting H. pylori from the toxicity of NaOCl. As shown in Figure 2D, the inhibition zone of H. pylori ΔkatA growth caused by NaClO was gradually reduced in response to increasing amounts (0.1–10.0 µg) of OMV\textsuperscript{wt} used for preincubation with NaClO. Taken together, our data indicated that H. pylori OMVs exhibiting KatA-dependent catalase activity successfully neutralized both H\textsubscript{2}O\textsubscript{2} and NaClO, and thus promoting bacterial survival when exposed to the bactericidal activity of ROS.

**FIGURE 2** OMVs carrying KatA hydrolyse/detoxify ROS and provide protection against ROS-mediated bacterial killing. (A) Comparison of catalase activity between OMVs derived from H. pylori 18943 wt and a bacterial lysate. An increased H\textsubscript{2}O\textsubscript{2} hydrolysis on a weight basis was detected with OMVs compared to bacterial lysate. (B) Determination of KatA-dependent catalase activity in OMVs. Deletion of KatA in two different strains of H. pylori, 18943 and P12 resulted in abolished catalase activity in OMVs from H. pylori ΔkatA mutant strains compared to OMVs derived from the wild type counterparts. In (A) and (B), catalase activity was presented as nmol of H\textsubscript{2}O\textsubscript{2} decomposed per mg of sample tested (mean ± SD; n = 3; *p < 0.05; **p < 0.01). (C) and (D). Helicobacter OMVs reduce the bactericidal activity of H\textsubscript{2}O\textsubscript{2} and NaClO, and thus promoted bacterial survival. In (C), H. pylori P12ΔkatA devoid of catalase activity was challenged with 1 mM H\textsubscript{2}O\textsubscript{2} preincubated with 40 µg/ml of OMV (equivalent to OMVs produced from 10\textsuperscript{8} CFU) (OMV\textsuperscript{wt}, OMV\textsuperscript{ΔkatA} or heat-inactivated OMVs; OMV\textsuperscript{hia}), In (D), H. pylori 18943ΔkatA devoid of KatA expression was exposed to 5% NaClO that had been pre-treated with 0.1–10.0 µg of OMV\textsuperscript{wt}. Pure H\textsubscript{2}O\textsubscript{2} and NaClO in PBS that were fully bactericidal were included as a positive control. In (C), the viability of bacteria was assessed by plating and counting colony forming units (CFU). Only KatA-containing OMV\textsuperscript{wt} was able to neutralize H\textsubscript{2}O\textsubscript{2} and promote survival of H. pylori P12ΔkatA from peroxidative killing. OMV lacking KatA (OMV\textsuperscript{ΔkatA} or heat-inactivated KatA (OMV\textsuperscript{katA\_lacking}) did not show any protection of whole bacteria (mean ± SD; n = 3; *p < 0.05; **p < 0.01). In (D), the bactericidal activity of NaClO was decreased by preincubation with increasing amounts of OMV\textsuperscript{wt} (equivalent to OMVs produced from 0.2 to 2 × 10\textsuperscript{8} CFU). The killing of H. pylori 18943ΔkatA by NaOCl was measured by the diameter of inhibition zone, which is the area without bacterial growth (mean ± SD; n = 3; **p < 0.01; ***p < 0.001).
**TABLE 1 | List of bacterial strains used in this study.**

| Bacterial strain<sup>a</sup> | Description<sup>b</sup> | Reference or source |
|-------------------------------|--------------------------|---------------------|
| H. pylori 18943 wt            | Wild type. Clinical isolate from a gastric antrum biopsy. | Culture Collection, University of Göteborg, Sweden (CCUG) |
| H. pylori CCUG18943ΔkatA     | Km<sup>+</sup>. Isogenic katA deletion mutant of CCUG18943 was constructed by replacement of katA with nptI. The strain is devoid of KatA expression. | Richter et al., 2016 |
| H. pylori P12 wt             | Wild type. Clinical isolate from a duodenal ulcer patient. | Schmitt and Haas, 1994 |
| H. pylori P12ΔkatA           | Cm<sup>+</sup>. Isogenic katA deletion mutant of P12 by cat replacement. The strain is devoid of KatA expression. | This study |

<sup>a</sup>Helicobacter pylori was grown on chocolate agar or in Brucella broth (Sigma-Aldrich, St. Louis, MO, United States) supplemented with 10% horse serum and 1% Vitox (Oxoid, Hants, United Kingdom) in microaerobic environment at 37°C. Liquid cultures were incubated at 200 rpm. <sup>b</sup>Concentrations of antibiotics used: 30 µg/ml Kan; 20 µg/ml cm.

**DISCUSSION**

*Helicobacter pylori* has evolved several virulence mechanisms for persistent colonization and infection in the gastric mucosa and this includes release of OMVs (Parker and Keenan, 2012). Here, we deciphered a novel role of OMVs in the pathogenesis of *H. pylori*; OMVs act as antioxidative particles via enrichment of KatA at the surface of vesicles. To the best of our knowledge, the current study is the first report regarding enrichment of KatA in *H. pylori* OMVs. Intriguingly, the *H. pylori* virulence factors OipA and HtrA have also been reported to be enriched in OMVs (Olofsson et al., 2010).

Production of toxic ROS, i.e., superoxide (O<sub>2</sub>•<sup>−</sup>), nitrogen oxide (NO), H<sub>2</sub>O<sub>2</sub>, and ClO<sup>−</sup> by human polymorphonuclear cells (PMNs) during the respiratory burst is an important component of the innate defense to eradicate phagocytosed pathogens (Yang et al., 2013). Despite *H. pylori* infection induces massive influx of neutrophils into the gastric mucosa and production of ROS, the pathogen expresses KatA to survive at the surface of vesicles. *H. pylori* is thus antiphagocytic and resistant against the respiratory burst-dependent killing (Ramarao et al., 2000; Wang et al., 2006). In contrast to bacterial cell-associated KatA, little is known regarding the KatA-dependent ROS resistance of OMVs.

We speculated that the accumulation of KatA in *H. pylori* OMVs, and thus higher catalase activity compared to bacterial cells (Figure 2A), may confer OMVs as an antioxidant cargo to protect bacteria from extracellular ROS of the respiratory burst. Bacterial interactions with PMNs result in an increase of extracellular H<sub>2</sub>O<sub>2</sub> and ClO<sup>−</sup> release by neutrophils that is ineffective, however, to efficiently eradicate non-phagocytosed *H. pylori* (Ramarao et al., 2000; Allen et al., 2005). In addition to KatA, other antioxidative proteins such as KatB and AhpC are also present in the OMV proteome (Mullaney et al., 2009; Olofsson et al., 2010). However, we found that the catalase activity of OMVs is solely attributed to KatA accumulation since the ability to hydrolyse H<sub>2</sub>O<sub>2</sub> was diminished in OMVs lacking KatA (Figure 2B). This could be due to the relatively low amount of KatB and AhpC compared to KatA in the *H. pylori* OMVs (Mullaney et al., 2009; Olofsson et al., 2010). Importantly, the KatA-dependent catalase activity of *H. pylori* OMVs is highly conserved among different strains (Figure 2B), further suggesting OMVs as important antioxidative particles.

In this study, we employed the direct H<sub>2</sub>O<sub>2</sub> and NaClO bactericidal assay as an *in vitro* extracellular ROS respiratory burst model. Of note, *H. pylori* KatA counteracts the oxidative damage of H<sub>2</sub>O<sub>2</sub> and ClO<sup>−</sup> via different mechanisms, which are through its catalase hydrolysis activity and oxidation of KatA methionine residues, respectively (Wang et al., 2006; Benoit and Maier, 2016). Our results demonstrated that *H. pylori* OMVs effectively neutralized ROS and rescued bacteria from lethal oxidative damage (Figures 2C,D).

The strategy to promote bacterial infection by virulence factor enrichment in OMVs has also been reported with other pathogens. *Bacteroides* spp. escapes from antibiotics by decorating their OMV surface with cephalosporinases (Stenz et al., 2015). *Aggregatibacter actinomycetemcomitans* utilizes OMVs enriched with leukotoxin to induce immune cell apoptosis (Bonnington and Kuehn, 2014). Our finding pioneered the idea of virulence factor enrichment in OMVs as a novel virulence mechanism of *H. pylori*. This is exemplified by KatA in OMVs that, in turn, contributes to the novel antioxidative role of *H. pylori* OMVs, and thus enhanced bacterial defense against host innate immune attacks. We speculate that, during infection in gastric mucosa, *H. pylori* releases OMVs enriched with KatA to decrease or deplete the surrounding extracellular ROS released from the oxidative burst of influxed PMNs. This will allow *H. pylori* to escape towards nearby infection sites with lower ROS, thereby facilitating bacterial survival and colonization. In conclusion, we have presented expanded insights on a novel potential virulence mechanism of *H. pylori* that provide additional knowledge regarding bacterial survival in a hostile PMN-rich environment.

**MATERIALS AND METHODS**

**Bacterial Strains and Growth Conditions**

Bacterial strains and growth conditions are listed in Table 1.

**Construction of *H. pylori* P12 ΔkatA Strain**

*KatA* gene (Genbank Accession Number: CP001217; encodes KatA) deletion was performed as previously
described (Richter et al., 2016). A linear katA-knockout construct containing cat (AY219687.1) was inserted between upstream and downstream flanking regions of the katA gene. Upstream flank was amplified from P12 gDNA using primers pair kat1F (5′-TCCCTGGAGCTGGTTGCAATA-3′) and kat1R (5′-CTTACGACTTGACCTAGAAGAGCTGAGTACAGCATTG-3′). Primers kat2F (5′-CAATGTTGCGCATGAAATGGGAAAACCTCTTGGGTCTTAC-3′) and kat2R (5′-CACCACAGTAATTTGGCCTAGTGTC-3′) were used to amplify downstream flank. Chlormphenicol resistance cassette was amplified from the gDNA of genomic DNA from strain J99sabB::cam using camF (5′-CAATGCTGTACAGCCTCTTC TAGGCTCAAGTGCTAAG-3′) and camR (5′-CGGTAAGAGCACCAGAGGT TTGCCATT CATGGCACCATGTC-3′) primers. The construct containing a chlormphenicol resistance cassette and flanking regions was created by overlap extension PCR. For the overlap reaction of each PCR product were used as templates with primer pair kat1F and kat2R. All of PCR reactions were carried by GoTaq polymerase (Promega) or Phusion Hot start DNA polymerase (Thermo Scientific), and MJPTC-200 thermal cycler (MJ Research). The overlap PCR product was purified by E.Z.N.A Cycle Pure or Gel Extraction kits (OMEGA Bio-Tek, Norcross, GA, United States), prior to transformed into P12 wt. The mutant was verified by sequencing (Eurofin MWG, Ebersberg, Germany).

**OM and OMVs Preparation**
To isolate OMVs, culture supernatants were concentrated using Vivaflow200 (Sartorius, Goettingen, Germany) and centrifuged at 165,000 × g (Olofsson et al., 2010). Pellets were separated by Histodenz (20–50%), and centrifugation at 200,000 × g and resuspended in phosphate buffered saline (PBS). OM was prepared from bacteria as described (Voss et al., 2014).

**Transmission Electron Microscopy (TEM)**
The localization of KatA at the surface of intact bacteria and OMVs was determined by purified rabbit anti-KatA polyclonal antibodies (pAb) labeled with 5 nm colloidal thiocyanate gold followed by TEM using negative staining (Olofsson et al., 2010).

**Estimation of KatA Concentrations and Catalase Enzymatic Assays**
Recombinant KatA (rKatA), OM, or OMVs sample were separated by SDS-PAGE followed by immunoblotting using anti-KatA pAb (Supplementary Figure S1) (Richter et al., 2016). Signal intensities generated from known amounts of rKatA were included as a standard curve for KatA estimation. Analysis was done by Image Lab software (Bio-Rad, Copenhagen, Denmark).

**H$_2$O$_2$ Bactericidal Assay**
H$_2$O$_2$ (1 mM) was preincubated with 40 µg/ml of OMV for 1 h at 37°C. Bacteria were resuspended in Brucella broth to an OD$_{600}$ of 0.1, and added to the OMV-treated H$_2$O$_2$. Mixtures were incubated for 3 h at 37°C, and plated on chocolate agar for 5 days at 37°C to enumerate the bacterial survival based on colony forming units (CFU). Control experiments were performed as described above by using only H$_2$O$_2$ without OMVs.

**Hypochlorous Acid-Based Disk Diffusion Sensitivity Assay**
A sterilized filter paper (5.4 mm in diameter) was saturated with 20 µl of 5% NaClO that had been pre-incubated for 3 h with OMVs. Bacterial colonies were resuspended in PBS and evenly spread on chocolate agar. Filter papers were placed on top of the agar, and plates were incubated at 37°C for 3 days. The diameter of inhibition zones was measured.

**Statistical Analysis**
Graph-Pad Prism® 7.0 (La Jolla, CA, United States) was used, and differences between groups or samples was considered statistically significant at $p < 0.05$.

**AUTHOR CONTRIBUTIONS**
AA, SL, Y-CS, and KR designed the study. AV, IO, MB, MA-K, and SL did experiments. KR, SL, and Y-CS wrote the manuscript. All authors have read and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01837/full#supplementary-material
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.