Moraxella catarrhalis outer membrane vesicles carry beta-lactamase and promote survival of *Streptococcus pneumoniae* and *Haemophilus influenzae* by inactivating amoxicillin

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ABSTRACT

Moraxella catarrhalis is a common pathogen found in children with upper respiratory tract infections, and in patients with chronic obstructive pulmonary disease during exacerbations. The bacterial species is often isolated together with *Streptococcus pneumoniae* and *Haemophilus influenzae*. Outer membrane vesicles (OMV) are released by *M. catarrhalis* and contain phospholipids, adhesins, and immunomodulatory compounds such as lipooligosaccharide. We have recently shown that *M. catarrhalis* OMV exist in patients upon nasopharyngeal colonization. As virtually all *M. catarrhalis* are β-lactamase positive, the goal of this study was to investigate whether *M. catarrhalis* OMV carry β-lactamase, and to analyze if OMV consequently can prevent amoxicillin-induced killing. Recombinant RH4 β-lactamase was produced and antibodies were raised in rabbits. Transmission electron microscopy, flow cytometry and Western blots verified that OMV carried β-lactamase. Moreover, enzyme assays revealed that *M. catarrhalis* OMV contained active β-lactamase. OMV (25 μg/ml) incubated with amoxicillin for 1 hr completely hydrolyzed amoxicillin at concentrations up to 2.5 μg/ml. In functional experiments, pre-incubation of amoxicillin (10xMIC) with *M. catarrhalis* OMV fully rescued amoxicillin-susceptible *M. catarrhalis*, *S. pneumoniae* and type b or non-typeable *H. influenzae* from β-lactam-induced killing. Our results suggest that the presence of amoxicillin-resistant *M. catarrhalis* originating from β-lactamase-containing OMV may pave the way for respiratory pathogens that by definition are susceptible to β-lactam antibiotics.

INTRODUCTION

After *Streptococcus pneumoniae* and non-typeable *Haemophilus influenzae* (NTHi), *Moraxella catarrhalis* is the most common cause of bacterial respiratory infections in humans. *M. catarrhalis* causes acute otitis media in children and exacerbations in adults with chronic obstructive pulmonary disease (COPD), but can also be found in patients diagnosed with sinusitis and laryngitis. *M. catarrhalis* resides in the palatine tonsils and invades epithelial cells in the respiratory tract (11, 14, 27, 37).

One important characteristic of *M. catarrhalis* is that the bacterium, like most other Gram-negative species, releases outer membrane vesicles (OMV). Over recent years OMV have been shown to contain several virulence factors allowing *M. catarrhalis* to evade the immune system and thus effectively colonize the host (31, 34, 37). Vesicles are formed when part of the bacterial outer membrane bulges out and pinches off, creating vesicles with sizes ranging between 50-250 nm (7, 19, 34, 36). OMV are composed of proteins and phospholipids found in the outer cell membrane, but can also contain certain periplasmic proteins closely associated with the membrane. Interestingly, OMV also contain immunomodulatory compounds, which enable bacteria to interact with the host immune system without requiring close contact (16). When we in detail analyzed *M. catarrhalis* OMV using a proteomics approach combining 2-dimensional SDS-PAGE and MALDI-TOF mass spectrometry, 57 different periplasmic or outer membrane proteins were identified (31).

While most *M. catarrhalis* clinical strains recovered before 1975 were susceptible to β-lactam antibiotics, strains isolated in the mid 1980s showed a rapid increase in resistance against β-lactams. It was found that these resistant isolates produced one of

two variants of a defined β-lactamase encoded by the genes *bro-1* or *bro-2* (38). However, since these alleles are considered to be >99 % identical (2), it was not surprising that any functional differences could not be found between strains. More than 97 % of all *M. catarrhalis* strains are today considered to be β-lactamase positive, and a majority of these (> 90 %) have the *bro-1* allele compared to the less frequently occurring *bro-2* allele (2, 13, 17).

M. catarrhalis is often found in mixed infections, and in up to 50 % of all *M. catarrhalis* clinical cultures either *S. pneumoniae* and/ or NTHi have also been identified (15). In contrast to *M. catarrhalis,* most *S. pneumoniae* and *H. influenzae* are susceptible to β-lactam antibiotics, that is, on a worldwide basis ≈14 % of *S. pneumoniae* and ≈21 % of NTHi clinical isolates are resistant to β-lactams (5). One possible advantage of the co-infection of *M. catarrhalis* with the two other bacterial species was convincingly shown in a mouse model, where β-lactamase producing *M. catarrhalis* conferred protection for *S. pneumoniae* against β-lactam antibiotics (12).

Since β-lactamase is found in the periplasm, we hypothesized that OMV might harbor β-lactamase and function as a long-distance delivery system to confer antimicrobial resistance for *M. catarrhalis*, but also for the other two bacterial species dwelling in the respiratory tract. We show the presence of β-lactamase in OMV isolated from *M. catarrhalis* and that OMV hydrolyze amoxicillin, and consequently rescue β-lactamase negative *H. influenzae* and *S. pneumoniae* from amoxicillin-induced killing.

MATERIALS AND METHODS

Bacterial strains, growth conditions and minimal inhibitory concentrations (MIC). Reference strains and clinical isolates from our department are shown in Table 1. *S. pneumoniae* American Type Culture Collection (ATCC) 6303 was grown in Todd Hewitt broth supplemented with 0.5 % Yeast Extract, and cultured on sheep blood agar plates. All other species were cultured on chocolate agar plates. *M. catarrhalis*, NTHi 772, and *H. influenzae* capsule type b (Hib) Eagan were grown in Brain-Heart Infusion (BHI) broth (Difco/Becton, Lawrence, KS) at 37 °C in 5 % CO_2. *H. influenzae* was grown with NAD and hemin (10 μg/ml for each). Bacteria were tested for β-lactamase activity using Nitrocefin disks (BioMérieux, Marcy L'Étoile, France). *H. influenzae* strains were additionally tested for β-lactamase status against penicillin G and cefaclor according to the manufacturers' instructions (Biodisk, Solna, Sweden). *E. coli* strains DH5α and BL21 were cultured in Luria Bertani (LB) broth at 37°C in a humid atmosphere containing 5 % CO_2.

To determine minimal inhibitory concentrations (MIC) for amoxicillin (Table 1), both E-tests (Biodisk,) and conventional colony counting (colony forming units; CFU) after incubation of bacteria in BHI broth (3) were used. A starting concentration of 10^7 CFU was used for determination of MIC in solution. *M. catarrhalis* with MIC ≤ 0.125 μg/ml amoxicillin were susceptible and > 0.125 μg/ml resistant (21). NTHi and *S. pneumoniae* with MIC ≤ 1 μg/ml and MIC ≤ 0.5 μg/ml amoxicillin, respectively, were considered susceptible.

Identification of *M. catarrhalis bro-1* and *bro-2* genes. The β-lactamase genes *bro-1* and *bro-2* were identified using PCR with primers

5’-TGTGCGAAGCTACCATAACACTGAGT-3’ and

5’-GGGGGCTTGTTGGGTCATAAATTTTTC-3’ followed by DNA sequencing. The *bro-1* and *bro-2* phenotypes are distinguished by a single amino acid change (aspartic acid to glycine) at position 294 that is caused by substitution of a single base pair (2).

Cloning of *bro*, recombinant protein expression and antibody production. In order to produce recombinant Bro for immunization purposes, the *bro-1* gene was isolated from genomic DNA obtained from *M. catarrhalis* strain RH4 (Table 1) using PCR-primers 5’-AGGAGATAATGATGGATCCCCGTCA-3’ and

5’-GGGATTTACCAAGCTTGGGCTGGGTGA-3’ containing *Bam*HI and *Hin*dIII restriction enzyme cleavage sites (underlined), respectively. The resulting PCR product (878 bp) was subsequently cloned into the vector pET26b (+) (Novagen, Darmstadt, Germany). To avoid presumptive toxicity, the vector was first transformed into *E. coli* strain DH5α and positive clones were selected using LB broth supplemented with 50 μg/ml kanamycin. Plasmids were further transformed into the expression host *E. coli* BL21 (DE3) and protein production was essentially performed as previously described by Singh *et al.* (32). Briefly, protein expression was induced by addition of 1 mM isopropyl-1-thio-β-D-galactoside (IPTG) to mid-log phase cultures (OD_{600} 0.6-0.8) for 3 hrs at 37°C. Subsequently, bacteria were sonicated, and proteins were purified using affinity chromatography (Histrap FF Crude; GE Healthcare Biosciences, Pittsburgh, PA) using His-tag elution buffer (50 mM Tris/HCl, 500 mM NaCl and 250 mM Imidazole, pH 7.5). After purification and protein concentration, a rabbit anti-β-lactamase (RH4) antiserum was prepared using an immunization protocol as described previously (23). Briefly, rabbits were immunized intramuscularly with 200 μg purified recombinant RH4 β-

lactamase, which had been emulsified in Complete Freud's adjuvant (Difco, Becton Dickinson, Franklin Lanes, NJ) and boosted on days 14 and 28 with the same doses of protein in incomplete Freund's adjuvant. Blood was drawn two weeks later and antibodies were purified against recombinant RH4 β-lactamase coupled to a CnBr-Sepharose column (WVR International, Leicestershire, UK). Antibodies were used in flow cytometry analysis and Western blot as described below.

Isolation of *M. catarrhalis* outer membrane vesicles (OMV). OMV were isolated using the Rosen method (30). Bacteria were grown in BHI broth overnight and after centrifugation the supernatants were filtered through 0.2 μm pore-size filters (Sartorius, Goettingen, Germany). Thereafter, the flow through was concentrated using 100,000 kDa Vivaspin centrifugal concentrators (Vivascience, Hannover, Germany). The precipitate containing the extracellular vesicles was collected by centrifugation for 1 hr at 100,000x*g*, and was washed with phosphate-buffered saline (PBS). Protein concentrations were determined by spectrophotometry using NanoDrop (NanoDrop Technologies, Wilmington, DE), and resulting OMV suspensions were checked on BHI agar to confirm that preparations were free of bacteria.

SDS-PAGE and Western blot analysis. To analyze whether OMV carry β-lactamase, OMV content was analyzed by 12 % SDS-PAGE. Gels were either stained with Bio-Rad Coomassie brilliant blue R-250 (Munich, Germany) or the proteins were transferred from the gel to an Immobilon-P membrane at 20 V overnight (Millipore, Bedford, MA). Following transfer, membranes were blocked with PBS containing 0.1 % Tween and 5 % milk powder for 1 hr. After several washes with 0.1 % Tween 20 (PBS-Tween), the membrane was incubated with rabbit anti-β-lactamase pAb diluted 1:200 in

PBS-Tween for 1 hr as described (23). After repeated washing steps, horseradish peroxidase (HRP)-conjugated goat anti-rabbit pAbs (DAKO, Glostrup, Denmark) diluted 1:1,000 were added for 1 hr. The membranes were then washed and developed using ECL Western blot detection reagents (Amersham Pharmacia Biotech, Uppsala, Sweden).

Flow cytometry analysis. To analyze the presence of β-lactamase in OMV, 3 μg OMV were fixed with 3.5 % formaldehyde for 15 min and then permeablized with Saponin (0.2 %) five min at RT. OMV were further incubated with recombinant RH4 rabbit anti-β-lactamase pAb diluted 1:5 in PBS-BSA (2.5 %) followed by addition of FITC-conjugated swine anti-rabbit pAb (DAKO). Between each labeling step, OMV were washed by ultracentrifugation at 100,000x*g* for 30 min. Samples were analyzed in an EPICS XL-MCL flow cytometer (Beckman Coulter, Hialeah, FL). A gate was set excluding signals ≤ 2.0 %.

Transmission electron microscopy (TEM). After fixation of the specimens, ultrathin sections were mounted on gold grids and subjected to antigen retrieval through the use of metaperiodate. Grids were floated on top of drops of immune reagents displayed on a sheet of parafilm. Free aldehyde groups were blocked with 50 mM glycine. Grids were thereafter blocked with 5 % (vol/vol) goat serum diluted in incubation buffer [0.2% bovine serum albumin-C in PBS, pH 7.6] for 15 min. OMV were then incubated overnight with primary antibodies (dilution 1:50-1:100) at +4°. Grids were washed in 200 μl incubation buffer and thereafter floated on drops containing the gold conjugate reagents of sizes 10 and 5 nm (diluted 1:10-1:20 in incubation buffer) for 1 hr at RT. After further washes in incubation buffer, sections were postfixed in 2 % glutaraldehyde and sections were washed in distilled water. They were then post-stained

with uranyl acetate and lead citrate, and examined under an electron microscope (Jeol JEM 1230; JOEL, Tokyo, Japan) operated at 60 kV accelerating voltage. Images were recorded with a Gatan Multiscan 791 CCD camera (Gatan, Pleasanton, CA).

Quantification of *M. catarrhalis* OMV β-lactamase activity. β-lactamase activity was determined using the chromogenic cephalosporin nitrocefin as previously described (3). Briefly, OMV preparations (0.3 μg/ml) were incubated with nitrocefin (0.5 mg/ml) (Oxoid, Thermo Scientific, Cambridge, UK) for 30 min at 37 °C in the dark. Following incubation, samples were spun down at 13,000x*g* for three min in order to remove larger proteins aggregated in the preparations, and the chromogen hydrolysis and subsequent color change of supernatants was determined immediately with NanoDrop at OD 485nm. The enzymatic activity was estimated using a standard curve where OD_{485} was related to the number of moles nitrocefin hydrolyzed. This was quantified using recombinant β-lactamase (VWR). Readings were thereafter converted to the number of mol nitrocefin hydrolyzed per min per mg protein. In order to compare β-lactamase activity in OMV compared to the parent strain, whole parent cell was heated to 95°C for 7 min in order to lyse bacteria. The nitrocefin hydrolyzation capacity of OMV versus parent strain lysate was then compared on a weight basis. Furthermore, in order to determine the localization of the β-lactamase in OMV, they were treated with 100 μg/ml proteinase K (Sigma Aldrich, St. Louis, MO) for 1 h at 50 °C. After deactivation with 10 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma Aldrich) and 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrocholoride (AEBSF) (USB, Cleveland, OH) samples were incubated with 0.02 % saponin and enzyme activity was measured as described above.

Measurement of β-lactamase induced amoxicillin hydrolysis. Amoxicillin concentrations were estimated using an agar diffusion method (18). *Sarcina lutea*, a highly β-lactam susceptible Gram-positive bacterium from the family of *Clostridiaceae* was plated on agar and allowed to dry. Perforations were made in the agar and samples containing OMV, which had been pre-incubated with amoxicillin for 1 hr at 37 °C, were added in duplicates. To allow diffusion of antibiotics into the agar as well as subsequent growth of bacteria, plates were left overnight at 37° C. The inhibitory zones (where no bacterial growth was observed) were measured and a standard curve was compiled.

Inactivation of amoxicillin by beta-lactamase transferred by OMV. Bacterial cultures were grown in a starting culture to a concentration of 10^6 -10^7 CFU/ml, followed by incubation with OMV at varying concentrations that had been pre-incubated at 37°C for 1 hr with amoxicillin at concentrations 10xMIC (Table 1). Cultures were grown in microtiter plates (Nunclon Surface, Thermo Fisher Scientific, Waltham, MA) at 37° C and 5 % CO_2. Bacterial growth was measured at OD_{600}, and at each time point triplicates of each culture were plated on chocolate agar or sheep blood plates and incubated overnight.

Statistical analysis. Statistical analyses were performed using GraphPad PRISM 5 (San Diego, CA). The Student's *t*-test was used to determine statistical differences for unpaired comparisons. All data is expressed as mean ± SEM, where *n* corresponds to the number of experiments performed. *, $p \leq 0.05$; **, $p \leq 0.01$; and ***, $p \leq 0.001$. Significant values were defined as $p \leq 0.05$.

RESULTS

***M. catarrhalis* OMV carry β-lactamase.** *Moraxella*-dependent resistance against amoxicillin was evaluated in a set of different strains (Table 1). E-tests were used to define MICs, and were also confirmed using conventional counting of colony forming units (CFU). To confirm that amoxicillin resistance in *M. catarrhalis* strains was due to the presence of β-lactamase genes, chromosomal analysis was included. Since the two alleles *bro-1* and *bro-2* have previously been found to encode for a β-lactamase in *M. catarrhalis*, clinical *M. catarrhalis* isolates (*n*=8) and two well-defined reference strains were screened by PCR and sequenced. As can be seen in Figure 1, 8 strains carried the *bro* gene, and of these only one was positive for *bro-2* that concurs with the assumed frequency of about 10 % in a defined population (3). The amoxicillin-resistant strains *M. catarrhalis* KR526 and RH4 with MIC ≥ 1 μg/ml were chosen for detailed analysis. For comparison, the amoxicillin-susceptible *M. catarrhalis* KR395 and Bc5 with MIC 0.064 and 0.032 μg/ml, respectively, were also included.

To verify that *M. catarrhalis* strains expressed β-lactamase, Western blots with total bacterial cell lysates and their corresponding released OMV were done using a rabbit anti-β-lactamase antiserum (Fig. 1B). Intriguingly, OMV originating from the β-lactamase positive *M. catarrhalis* KR526 contained β-lactamase. This was in contrast to OMV isolated from the β-lactamase negative strain *M. catarrhalis* Bc5 that did not contain any β-lactamase. Total cell lysates *M. catarrhalis* strains RH4 and KR395 were used as additional positive and negative controls, respectively. Moreover, our recombinant RH4 β-lactamase produced in *E. coli* was included as a positive control.

The presence of β-lactamase in OMV was also verified by flow cytometry after permeabilization with saponin (Fig. 1C). *M. catarrhalis* KR526 vesicles contained more

β-lactamase as compared to Bc5 when analyzed with the anti-β-lactamase antiserum followed by incubation with FITC-conjugated secondary antibodies. A clear shift (increased fluorescence intensity) was observed when OMV were analyzed by flow cytometry. However, β-lactamase was not detected in OMV analyzed in the absence of saponin (not shown) suggesting that β-lactamase mainly was located inside the vesicles. Transmission electron microscopy (TEM) with gold-labelled anti-β-lactamase pAb further showed the presence and absence of β-lactamase in KR526 and Bc5 OMV, respectively (Fig. 1D). Taken together, these experiments demonstrated that OMV derived from amoxicillin-resistant and β-lactamase positive *M. catarrhalis* also contained β-lactamase.

β-lactamase positive *M. catarrhalis* OMV hydrolyze amoxicillin. To determine the β-lactamase activity in our OMV preparations, the chromogenic substrate nitrocefin was used. OMV from four clinical *M. catarrhalis* strains as well as lysates from their parent bacteria were analyzed. The β-lactamase activity was expressed as the number of moles nitrocefin hydrolyzed per minute per mg protein (Fig. 2A). The two β-lactamase-positive strains *M. catarrhalis* KR526 and RH4 were confirmed, as well as the β-lactamase-negative strains *M. catarrhalis* Bc5 and KR395. Interestingly, although a higher MIC of amoxicillin was required for *M. catarrhalis* RH4, OMV from KR526 were shown to have the highest β-lactamase enzyme content on a weight basis. *M. catarrhalis* KR526 was thus selected for further experiments. However, it was also determined that there was no significant difference between the enzyme content of OMV and their parent bacteria. This suggested that β-lactamase was not enriched in vesicles.

After quantifying the β-lactamase content of OMV, the precise localization of β-lactamase was determined. OMV preparations were consequently pre-treated with proteinase K to digest β-lactamase associated with the outer membrane of the vesicles. OMV were thereafter treated with saponin to permeabilize OMV. Proteinase K treated OMV were found to have approximately the same enzymatic activity as non-treated ones. When OMV were additionally treated with saponin the β-lactamase activity increased. As a control, OMV were first treated with saponin and subsequently proteinase K in order to verify that proteinase K digested free β-lactamase. These results suggest that only a minor portion of β-lactamase was associated with the membrane of the vesicles, but most of the β-lactamase was found inside the vesicles.

The biological β-lactamase activity in the OMV preparations was determined by an antibiotic bioassay. β-lactamase-positive and -negative OMV were pre-incubated for 1 hr with amoxicillin and thereafter antibiotic concentrations were quantified by the ability to hydrolyze the highly amoxicillin-susceptible indicator bacterium *Sarcina lutea*. Varying concentrations of β-lactamase positive OMV from *M. catarrhalis* KR526 were incubated with amoxicillin (range 1.25-10 μg/ml) (Fig. 3A). OMV at 25 μg/ml were found to completely hydrolyze amoxicillin at concentrations up to 2.5 μg/ml, whereas a partial hydrolysis was observed at ≥ 5 μg/ml amoxicillin and 25 μg/ml OMV. In contrast to β-lactamase positive *M. catarrhalis* KR526 OMV, the β-lactamase negative OMV from KR395 did not hydrolyze amoxicillin, that is, no differences were found in the control with amoxicillin as compared to samples with amoxicillin pre-incubated with OMV deficient in β-lactamase (Fig. 3B). For comparison, samples pre-incubated with β-lactamase positive OMV (25 μg/ml) from KR526 completely hydrolyzed amoxicillin

concentrations up to 2.5 μg/ml. To summarize, our results indicated that OMV from *M. catarrhalis* carrying β-lactamase were able to hydrolyze and thus deactivate amoxicillin in a dose-dependent manner.

Amoxicillin-susceptible *M. catarrhalis* is protected against amoxicillin by β-lactamase-carrying OMV derived from another *M. catarrhalis* strain. Since several strains of *M. catarrhalis* have been shown to reside in one individual (2), β-lactamase positive *M. catarrhalis* OMV may play an important role in co-infections with β-lactamase negative *M. catarrhalis*. To investigate this phenomenon, amoxicillin with or without pre-incubation with *M. catarrhalis* OMV was analyzed against the β-lactamase negative and thus amoxicillin-susceptible *M. catarrhalis* KR395. Pre-incubation of amoxicillin (1 μg/ml) with OMV derived from KR526 carrying β-lactamase rescued bacteria (10^7 CFU/ml) from amoxicillin-induced killing, and resulted in bacterial growth comparable to the control without amoxicillin as revealed by optical density measurements (Fig. 4A).

To determine the number of viable bacteria upon incubation with amoxicillin with or without OMV, CFU were also counted (Fig. 4B). The results confirmed optical density measurements and in these experiments it was even more evident that amoxicillin-susceptible *M. catarrhalis* KR395 was rescued by OMV KR526 that were loaded with β-lactamase. In contrast, the protective effect was not seen with *M. catarrhalis* incubated with amoxicillin that had been pre-treated with β-lactamase negative OMV from *M. catarrhalis* Bc5 as demonstrated by changes in absorbance over time (Fig. 4C). Vesicles without β-lactamase neither inhibited nor promoted bacterial growth in the absence of

amoxicillin. Thus, OMV containing β-lactamase hydrolyzed amoxicillin and promoted growth of non-β-lactamase producing and hence amoxicillin-susceptible *M. catarrhalis*.

***M. catarrhalis* OMV containing β-lactamase rescue amoxicillin-susceptible non-typable *H. influenzae* (NTHi) and *S. pneumoniae*.** To reveal whether OMV isolated from β-lactamase-producing *M. catarrhalis* also protect other respiratory pathogens from amoxicillin, the susceptible NTHi 722 (10^7 CFU/ml) was exposed to amoxicillin (2 μg/ml) that had been pre-treated with OMV KR526 containing β-lactamase. A significant difference in growth rate could be seen between cultures exposed to amoxicillin that were pre-incubated with β-lactamase-carrying OMV and the control with amoxicillin only (Fig. 5A). This was also confirmed when CFU were determined (Fig. 5B). Finally, when NTHi 722 was incubated with amoxicillin exposed to β-lactamase negative OMV Bc5, no difference was found as compared to amoxicillin-treated NTHi 722 in the absence of OMV (Fig. 5C). The addition of OMV in the absence of amoxicillin did not interfere with bacterial growth.

The incidence of encapsulated *H. influenzae* type b (Hib) has decreased in the Western hemisphere due to successful vaccine campaigns, but still Hib is a significant problem in certain developing countries. We therefore also included Hib in our study. In parallel with NTHi, β-lactamase-containing *M. catarrhalis* OMV rescued Hib to the same extent as NTHi in the presence of amoxicillin (Fig. 5D).

It is a well-known fact that *S. pneumoniae* is significantly more susceptible to amoxicillin compared to *M. catarrhalis* and NTHi (9). In parallel with *M. catarrhalis* (Fig. 4) and NTHi (Fig. 5), *S. pneumoniae* ATCC 6303 (10^6 CFU/ml) was rescued from amoxicillin-induced killing when amoxicillin (1 μg/ml) was pre-incubated with β-

lactamase carrying *M. catarrhalis* OMV (Fig. 6A). This protective effect was also verified by determination of CFU (Fig. 6B), but was not observed with amoxicillin preparations pre-incubated in the presence of β-lactamase negative OMV (Fig. 6C). *S. pneumoniae* incubated with β-lactamase positive or negative OMV in the absence of amoxicillin did not interfere with bacterial growth. Taken together, OMV derived from β-lactamase-producing *M. catarrhalis* hydrolyzed amoxicillin resulting in significantly increased survival of NTHi, Hib and finally pneumococci that all were susceptible to amoxicillin.

DISCUSSION

In this study we have shown that OMV from *M. catarrhalis* carry β-lactamase at high concentrations and that these vesicles are able to protect amoxicillin-susceptible *M. catarrhalis* as well as *H. influenzae* and pneumococci against amoxicillin-induced killing. It has previously been demonstrated that β-lactamase, along with other proteins as well as DNA, can be transferred between different strains of *Pseudomonas aeruginosa* (1, 4, 26), a pathogen found in, for example, patients with cystic fibrosis (CF) (33). Thereby, β-lactamase can be shared between strains, and thus obliterate the need for each strain to carry its own resistance gene. Using electron microscopy and enzyme studies Ciofu *et al.* showed that β-lactamase was packaged inside the secreted *P. aeruginosa* OMV (4). In the present study, we identified functional β-lactamase inside *M. catarrhalis* OMV as judged by flow cytometry and permeabilization with saponin. TEM supported our observations with flow cytometry. Thus, antibiotic resistance could indeed be conferred to other *M. catarrhalis* strains by the aid of OMV hydrolyzing amoxicillin. We also determined that β-lactamase was localized inside the OMV, and thus derived from the periplasm of the

parent bacteria. This is in accordance with a study by Bootsma *et al* (1999) where it was suggested that the β-lactamase activity was found in the inner leaflet of the outer membrane facing the periplasmic compartment of *M. catarrhalis*. Our results further support the notion that amoxicillin can traverse over the outer membrane of the OMV into the lumen, and that compartment would be the localization of amoxicillin hydrolyzation.

Acute otitis media has previously been associated with a complex polymicrobial state. The explanation for this common phenomenon of mixed infections, however, largely remains unclear. *S. pneumoniae* and *H. influenzae* are often found as co-pathogens in infections with *M. catarrhalis,* and the reason for this has been speculated over (15). Budhani *et al.* found that *S. pneumoniae* growing in biofilm in the presence of β-lactamase positive *M. catarrhalis* were protected against killing when treated with amoxicillin (3). Additionally, *in vivo* experiments showed that mice infected intranasally with pneumococci and treated with amoxicillin or penicillin died from pneumococcal pneumonia if they were co-infected with β-lactamase-producing *M. catarrhalis* (12). In contrast, this effect was not found when mice were co-inoculated with β-lactamase negative *M. catarrhalis*. The transfer of β-lactam resistance is thus thought to be an important advantage for bacteria co-inhabiting with β-lactamase positive *M. catarrhalis*. Interestingly, we found that β-lactamase was transferred from *M. catarrhalis* by means of OMV protecting *S. pneumoniae* and *H. influenzae* against amoxicillin, suggesting this to be a novel mechanism for conveying antimicrobial resistance.

Several secretion systems are used by bacterial species in order to invade their hosts and cause infection. Both type III and IV secretion systems allow bacteria to deliver

proteins directly into the cytoplasm of the host cell. OMV have been identified as a novel secretion system, where no contact is required between the invading bacteria and its host. OMV adhere and fuse with host cells at lipid rafts in cell membranes, thus allowing them to deliver various bacterial factors (36, 37). In this way OMV can secrete virulence factors from a distance to host cells, still causing infection but staying clear of the host immune response. Several virulence factors of the common pathogen *M. catarrhalis* are packaged into OMV, such as MID, UspA1/A2, CopB, OMP CD, OMP E and lipooligosaccharides (LOS) (31, 35, 37). We recently showed that OMV secreted from *M. catarrhalis* interact with human tonsillar B cells (37). Through induction of B-cell receptor clustering and TLR signaling, OMV were found to bind and activate B cells. The superantigen MID (8, 24) and DNA associated with the OMV membrane were found to be essential for maximal B cell activation in a non-immunogenic fashion.

We have previously shown that the *M. catarrhalis* OMV also protect *H. influenzae* against complement-mediated attacks (34). The *M. catarrhalis* proteins UspA1/A2 bind and deplete the third component of the complement system (C3), an essential protein of the complement cascade in serum (22, 39). Furthermore, it was established that OMV containing UspA1 and A2 interfered with the complement cascade and thereby increased the survival of *H. influenzae.* Such a symbiotic relationship between two common pathogens might also be of benefit to *M. catarrhalis,* since the promotion of the co-pathogen *H. influenzae* may cause increased inflammation leading to an upregulation of epithelial cell surface receptors. This may also facilitate the adherence of *M. catarrhalis*, and thus potentiate infection (34).

Conferment of β-lactamase in OMV is a novel mechanism by which *M. catarrhalis* not only enhances survival of its own species but also promotes infection of co-inhabiting pathogens such as *H. influenzae* and *S. pneumoniae*. Considering the problem with the current global spread of antibiotic resistance, it is of highest importance to elucidate all possible mechanisms by which bacteria can cause infection through avoidance of antimicrobial agents. OMV are an interesting novel target for innovative therapies in combination with conventional antibiotics during treatment of chronic and acute bacterial infections.

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FIGURE LEGENDS

FIG. 1. *Amoxicillin resistant* M. catarrhalis *strains produce OMV containing β-lactamase.* (A) Eight *M. catarrhalis* strains out of 10 were positive for the *bro*-gene (522 bp) as revealed by PCR analysis. *Bro*-alleles were not found in strains KR395 and Bc5. (B) *M. catarrhalis* OMV and total bacterial lysates (10 μg each) were subjected to SDS-PAGE (left panel) followed by detection of β-lactamase (35 kDa) by Western blot (right panel). (C) Flow cytometry using antiserum raised against recombinant RH4 β-lactamase. (D) Gold-labeled anti-β-lactamase antibodies confirmed the presence of β-lactamase in transmission electron microscopy (TEM). In (B), lysates of whole *M. catarrhalis* RH4 and KR395 bacteria were used as positive and negative controls, respectively. Recombinant RH4 β-lactamase (0.6 μg) was also included. The upper band represents the recombinant protein at a size of 37.7 kDa. The lower band most likely results from N-terminal degradation. The His-tag located at the C-terminal end was not affected by degradation since it was possible to purify the recombinant β-lactamase using affinity chromatography. In (C), the arrow shows a positive shift with β-lactamase containing OMV KR526, whereas OMV from Bc5 were negative. OMV without the β-lactamase antiserum (black) is compared to OMV incubated with β-lactamase antiserum (white). In (D), the bar represents 100 nm.

FIG. 2. M. catarrhalis *OMV contain enzymatically active β-lactamase.* The β-lactamase content of whole cell lysate and OMV was analyzed from four different *M. catarrhalis* strains (A). The β-lactamase enzyme was found on the inside of the OMV (B). The β-lactamase activity was quantified by the ability of the enzyme to hydrolyze the β-lactam

nitrocefin, leading to a change in absorbance from OD_{380} to OD_{485} as determined by spectrophotometry. In (A) OMV contained approximately the same β-lactamase content as whole cell lysates. In (B), β-lactamase enzyme or OMV were treated with proteinase K (100 μg/ml) and/or saponin (0.2 %), and enzyme content was subsequently determined. As a negative control, OMV were first treated with saponin followed by proteinase K. The β-lactamase content was expressed as number of moles nitrocefin hydrolyzed per minute per mg OMV.

FIG. 3. *β-lactamase-carrying* M. catarrhalis *OMV hydrolyze amoxicillin*. Amoxicillin-induced killing at 1.25-10 μg/ml amoxicillin was gradually reduced with increasing concentrations (0-50 μg/ml) of β-lactamase-containing OMV. Amoxicillin concentrations were determined by measuring inhibitory growth zones of the β-lactam susceptible bacterium *Sarcina lutea* (A). β-lactamase positive and negative *M. catarrhalis* OMV at 25 μg/ml were incubated with increasing amoxicillin concentrations (B). The data are presented as means and the standard error of means (SEM) of at least three independent experiments. ***, $p \leq 0.001$.

FIG. 4. *β-lactamase positive* M. catarrhalis *OMV protect amoxicillin-susceptible* M. catarrhalis *strains from being killed by amoxicillin.* The β-lactamase susceptible *M. catarrhalis* KR935 (10^7 CFU/ml) was grown with amoxicillin (1 μg/ml) that had been pre-incubated in the presence of 25 μg/ml β-lactamase positive (A, B) or negative OMV (B, C). β-lactamase positive and negative OMV were isolated from *M. catarrhalis* KR526 (β-lac+) and Bc5 (β-lac-), respectively. Growth was expressed either as relative

growth compared to starting concentrations measured as absorbance (OD_{600}) (A, C), or as colony forming units (CFU) (B). Mean values and SEM of at least three independent experiments are shown. *, $p \leq 0.05$.

FIG. 5. *Vesicles from β-lactamase-positive* M. catarrhalis *protect NTHi against amoxicillin.* The amoxicillin-susceptible NTHi 772 (A-C) or Hib KR124 (D) (10^7 CFU/ml) was grown with amoxicillin (2 μg/ml) that had been pre-incubated with either 25 μg/ml β-lactamase positive (A, B) or negative OMV (B, C). OMV were isolated from *M. catarrhalis* KR526 and Bc5 that were β-lactamase positive and negative, respectively. Growth was expressed either as relative growth compared to starting concentrations measured as absorbance at OD_{600} (A, C) or as CFU (B, D). The results are shown as means and SEM of at least three independent experiments. *, $p \leq 0.05$; and ***, $p \leq 0.001$.

FIG. 6. M. catarrhalis *β-lactamase containing OMV protect* S. pneumoniae *from being killed by amoxicillin.* The amoxicillin-susceptible *S. pneumoniae* ATCC 6303 (10^6 CFU/ml) was grown with amoxicillin (1 μg/ml) either pre-incubated with 25 μg/ml β-lactamase positive (A, B) or negative OMV (B, C). OMV were isolated from *M. catarrhalis* KR526 (β-lac+) and Bc5 (β-lac-). Growth was expressed either as relative growth compared to starting concentrations measured as absorbance (OD_{600}) (A, C) or as colony forming units (CFU) (B). The data are presented as means and the standard errors of at least three independent experiments are indicated. *, $p \leq 0.05$; **, $p \leq 0.01$; and ***, $p \leq 0.001$.

TABLE 1. Clinical isolates and reference strains. The presence of the *bro1* or *bro2* genes encoding *M. catarrhalis* β-lactamase is also shown.

Clinical isolate/ strain	Site of isolation	Age	Gender	Clinical manifestation	MIC (μg/ml)	Amoxicillin susceptibility	β-lac. status
M. catarrhalis							
Bc5	Nasopharynx (reference strain)				0.032	Sensitive	Negative
RH4	Blood (reference strain)				2.0	Resistant	Positive
KR395	Tympanic cavity	66 yrs	Female	Cough	0.064	Sensitive	Negative
KR492	Nasopharynx	9 mon	Male	Otitis media	0.50	Resistant	Positive
KR493	Nasopharynx	4 yrs	Female	Recurring fever	16.0	Resistant	Positive
KR522	Nasopharynx	35 yrs	Male	Unknown	8.0	Resistant	Positive
KR523	Nasopharynx	79 yrs	Male	Unknown	8.0	Resistant	Positive
KR526	Nasopharynx	33 yrs	Male	Cough, sore throat	1.0	Resistant	Positive
KR542	Nasopharynx	2 mon	Male	Unknown	6.0	Resistant	Positive
KR923	Nasopharynx	4 yrs	Male	Otitis media	3.0	Resistant	Positive
H. influenzae							
NTHi 722	Nasopharynx (reference strain)				0.19	Sensitive	Negative
Hib Eagan	Reference strain				0.50	Sensitive	Negative
S. pneumoniae							
ATCC 6303	Reference strain				0.094	Sensitive	Negative

*Minimal Inhibitory Concentration (MIC) as determined by E-test.