Received: 12 April 2022 | Revised: 12 August 2022 | Accepted: 12 September 2022

DOI: 10.1002/jex2.61

RESEARCH ARTICLE

Nutrient smuggling: Commensal gut bacteria-derived extracellular vesicles scavenge vitamin B12 and related cobamides for microbe and host acquisition

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Funding information
Royal Society, Grant/Award Number: INF\R2\180062; Biotechnology and Biological Sciences Research Council, Grant/Award Numbers: BB/R012490/1, BB/S002197/1, BBS/E/F/000PR10346, BBS/E/F/000PR10353, BBS/E/F/000PR10355

Abstract

The processes by which bacteria proactively scavenge essential nutrients in crowded environments such as the gastrointestinal tract are not fully understood. In this context, we observed that bacterial extracellular vesicles (BEVs) produced by the human commensal gut microbe *Bacteroides thetaiotaomicron* contain multiple high-affinity vitamin B_{12} binding proteins suggesting that the vesicles play a role in micronutrient scavenging. Vitamin B_{12} belongs to the cobamide family of cofactors that regulate microbial communities through their limited bioavailability. We show that *B. thetaiotaomicron* derived BEVs bind a variety of cobamides and not only deliver them back to the parental bacterium but also sequester the micronutrient from competing bacteria. Additionally, Caco-2 cells, representing a model intestinal epithelial barrier, acquire cobamide-bound vesicles and traffic them to lysosomes, thereby mimicking the physiological cobalamin-specific intrinsic factor-mediated uptake process. Our findings identify a novel cobamide binding activity associated with BEVs with far-reaching implications for microbiota and host health.

KEYWORDS

bacterial extracellular vesicles, bacteroides, cobamides, microbe–host interaction, nutrient uptake, vitamin B_{12}

1 | INTRODUCTION

Vitamin B_{12}, which is also known as cobalamin or just B_{12}, is an essential dietary nutrient that belongs to the broader cobamide family of cofactors and coenzymes (Kennedy & Taga, 2020; Smith et al., 2018). Cobamides (Figure 1a) are structurally complex molecules whose biological synthesis is reflected by an equally complex biosynthetic pathway, which has never made the transition into eukaryotic systems (Bryant et al., 2020; Warren et al., 2002). Humans acquire B_{12} from foods such as meat, eggs, fish and dairy products where the B_{12} is produced by bacteria in the intestine of ruminants (meat and dairy products) or by bacteria that live symbiotically with algae (fish) (Smith et al., 2018; Stabler & Allen, 2004; Watanabe et al., 2013). Cobalamin is the cobamide that is specifically utilized by humans rather than any of the 15 or so other analogue forms found in nature (Figure 1a) (Hoffmann et al., 2000; Kennedy & Taga, 2020). These cobalamin analogues differ from B_{12} mainly in the nature of the lower

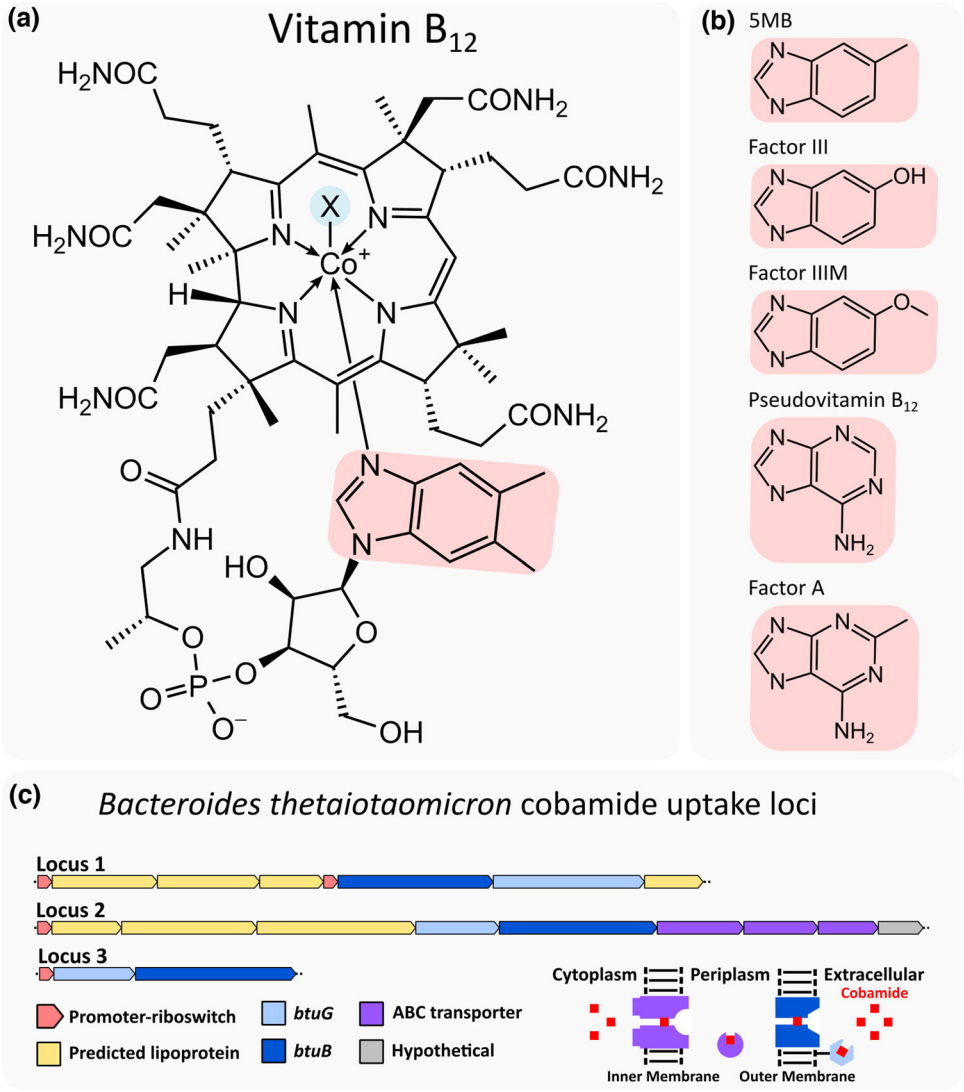


FIGURE 1 Structure of vitamin B_{12} and its analogues used in this work. (a) Structure of vitamin B_{12} with the upper ligand (X) highlighted in blue and the lower ligand highlighted in red. The main functional upper ligands include X = 5′-deoxyadenosyl and methyl groups although the position can also be occupied by hydroxy or cyano groups. (b) The lower ligands found in B12 analogues used in this work.
5 MB = Coβ-cyano-(5-methylbenzimidazolyl)-cobamide; Factor III = Coβ-cyano-(5-hydroxybenzimidazolyl)-cobamide; Factor IIIM = Coβ-cyano-(5-methoxybenzimidazolyl)-cobamide; Pseudovitamin B_{12} = Coβ-cyano-adeninyl-cobamide; Factor A = Coβ-cyano-(2-methyladeninyl)-cobamide. (c) Representation of the three *B. thetaiotaomicron* cobamide uptake loci discussed in this work. Each operon contains a predicted cobalamin regulated riboswitch. The outer membrane uptake components are represented in blue while the predicted inner membrane transporters are shown in purple with a schematic representation of these shown at bottom right. *btuG* encodes for the outer membrane anchored binding component; *btuB* encodes for the outer membrane spanning transporter.

nucleotide base of the molecule (Figure 1b) and appear to have limited biological activity in eukaryotic systems (Sokolovskaya et al., 2021; Sokolovskaya et al., 2020). The specificity for cobalamin within humans is determined by a highly evolved and intricate absorption process, whereby a high-affinity B_{12}-glycoprotein called gastric intrinsic factor selectively binds cobalamin within the small intestine, prior to absorption of the complex in the distal ileum (Gherasim et al., 2013; Smith et al., 2018). After release of the B_{12} via lysosomal degradation within gut epithelial cells, the nutrient is distributed in the blood by another highly specific cobalamin-binding protein called transcobalamin II (Nexo & Hoffmann-Lücke, 2011). However, despite the high affinity for B_{12}, humans have been reported to acquire other non-functional corrinoids although the means of that acquisition has not been determined (Kanazawa & Herbert, 1983).

The human digestive tract microbiome makes significant quantities of cobamides, but since the highest microbial density is found in the colon, it is not thought to provide a significant source of the nutrient to the host (Allen & Stabler, 2008). In fact, around a third of the bacterial species that make up the human gut microbiota house the necessary enzymes to make cobamides and these producers support other B_{12}-requiring bacteria either through symbiotic relationships or mutualistic interactions.

Cobamides, therefore, act as key modulators of gut microbial ecology (Degnan et al., 2014; Magnúsdóttir et al., 2015; Shelton et al., 2019). To ensure the acquisition of the nutrient from their environment bacteria have evolved multiple uptake systems with some converting it into an alternative cobamide form specific to their use, thereby contributing to the large number of variant cobamides (Escalante-Semerena, 2007; Mok et al., 2020; Sokolovskaya et al., 2020). Consequently, some bacteria pose a threat to their host by competing for and depleting cobalamin required by their human host in the small intestine (Albert et al., 1980). Bacteria within the large intestine appear to produce largely cobalamin analogues (Allen & Stabler, 2008), which are potentially deleterious to host health and falsely elevate readings of circulating B_{12} in blood (Allen & Stabler, 2008; Kolhouse et al., 1978; Stabler et al., 1991).

B. thetaiotaomicron is a prominent commensal gut bacterium and is a useful model organism for investigating microbe–host interactions (Ryan et al., 2020; Xu et al., 2003). The bacterium lacks the biosynthetic pathway for vitamin B_{12} production yet is dependent on the nutrient for growth by virtue of relying on a B_{12}-dependent methionine synthase (Xu et al., 2003). To acquire cobamides, the bacterium employs the *btuBFCD* B_{12} transport uptake/utilization system, where *btuB* encodes for an outer membrane permease, and *btuFCD* an inner membrane ABC type transporter (Goodman et al., 2009; Postle & Kadner, 2003). The bacterium has three genetically distinct uptake loci involved in scavenging different cobamides (Figure 1c) that also encode for a high-affinity B_{12}-binding outer membrane lipoprotein, BtuG (Degnan et al., 2014; Goodman et al., 2009; Wexler et al., 2018). As *B. thetaiotaomicron* produces significant quantities of extracellular vesicles that contain outer membrane lipoproteins, we reasoned that the B_{12}-binding lipoproteins may be present on these vesicles and could play a role in cobamide acquisition (Jones et al., 2020; Valguarnera et al., 2018).

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and culture conditions

B. thetaiotaomicron VPI-5482 strains and mutants used are outlined in Table S1. Growth was carried out in an anaerobic cabinet at 37°C. Cultures were maintained in BHIH media – Brain Heart Infusion (Oxoid) supplemented with 4-μM hemin (Sigma-Aldrich). Bacteroides defined media r3 (BDMr3) (100-mM potassium phosphate (pH 7.4); 15-mM NaCl; 8.5-mM $(NH_4)_2SO_4$; 4-μM hemin; 30-mM glucose; 0.2-mM *L*-histidine; 6-μM menadione (vitamin K3); 0.1-mM $MgCl_2$; 50-μM $CaCl_2$; 1.4-μM $FeSO_4$ supplemented with 100-nM vitamin B_{12} or 200-μM *L*-methionine, equilibrated in the anaerobic cabinet for at least 24 h followed by the addition of 4-mM *L*-cysteine hydrochloride) was developed for the experiments based on the media described previously (Martens et al., 2008)

2.2 | Bacterial extracellular vesicle purification

BtBEV preparation method was modified from that described (Jones et al., 2020). The cells were cultured from frozen stocks in 5-ml BHIH media supplemented with *L*-methionine for 16 h to an optical density at 600 nm (OD_{600}) of 1 to 2. These were sub-cultured at 1:100 dilution into BDMr3 supplemented with *L*-methionine or vitamin B_{12} and grown for 5 h. The cultures were used to inoculate 200 ml or 500 ml of the same media at 1:100 dilution and grown for 20 h. The cells were spun down at 6600 g for 1 h. Immediately, the supernatant was vacuum-filtered using a 0.22-μm filter to remove any left-over bacteria. The supernatant containing the vesicles was concentrated to around 5 ml using crossflow filtration (Vivaflow 50R; 100 kDa) and washed five times with 100-ml phosphate buffered saline (PBS), concentrating to around 5 ml between washes. The vesicles were subsequently spun at 15,000 g for 20 min collecting the supernatant to remove any precipitate. The vesicles were then filter-sterilized using a 0.22-μm syringe filter and quantified using nanoparticle tracking analysis.

2.3 | Nanoparticle tracking analysis

Particle quantification was carried out using a ZetaView instrument (Particle Metrix) with ZetaView (version 8.05.12 SP1) software running a 2 cycle 11 position high frame rate analysis at 25°C. Sample dilutions were carried out in ultra-pure water prior to analysis to fit within an optimal detection range. Camera control settings: 80 sensitivity; 30 frame rate; 100 shutter. Post-acquisition parameters: 20 min brightness; 2000 max area; 5 min area; 30 tracelength; 5 nm/class; 64 classes/decade.

2.4 | *Bacteroides thetaiotaomicron* cobamide bioassay

The methodology is outlined in Figure 1a. VPI-5482 *B. thetaiotaomicron* cells were cultured from frozen stocks in 5-ml BHIH media supplemented with *L*-methionine for 24 h to an OD_{600} of 1 to 2. The cells were then sub-cultured at 1:100 dilution into BDMr3 media lacking vitamin B_{12} or *L*-methionine and cultured for 24 h leading to the depletion of *L*-methionine within the

cells. The assay was set up in a 24-well tissue culture treated plate (Greiner) using three rows as triplicates and the fourth row for positive and negative controls. BDMr3 media (1 mL) lacking vitamin B_{12} or *L*-methionine was inoculated with 10 μl of depleted culture and incubated with 10 μl of test sample (BtBEVs used at 10^{10} ml-1 final concentration). Sterile gas permeable adhesive seals (Fischer Scientific) were used to seal the plates to limit evaporation. Plates were incubated for 24 h, OD_{600} readings were taken and adjusted to a percentage using the control well. This adjustment was required as even with adhesive seals, some evaporation was observed. This was likely due to the limitations of the experiments carried out in anaerobic cabinets containing humidity controls.

2.5 | BtBEV saturation

BtBEVs were saturated by incubating 10^{12} ml^{-1} BtBEVs with 0.5-μM cobamide or fB12. Five hundred microlitres of saturated vesicles were then concentrated using Vivaspin 500 100-kDa cut-off spin columns and washed five times with 480-μl PBS. Columns were spun at 12,000 g for 15 min discarding the supernatant between each step. The final sample was quantified using ZetaView particle analyzer.

2.6 | Cobamide isolation and purification

The cobalamin analogues described in this work were isolated, purified and characterized as previously described (Krautler et al., 1988; Krautler et al., 1987).

2.7 | Mutant strain generation

Generation of the *B. thetaiotaomicron* VPI-5482 Δ*btuG2* strain was carried out using an adapted method (García-Bayona & Comstock, 2019). Plasmid for the crossover was generated by inserting a fragment generated by overlap extension PCR using primers outlined in Table S2. Briefly, primers corresponding to upstream (Up), or downstream (Down) fragments were used to amplify fragments upstream and downstream of the target gene. These fragments were used in the overlap PCR reaction generating the insert. End primers contained engineered restriction enzyme sites for *BamHI* (Up) and *PstI* (Down), which were used to insert the fragment into pLGB13 (García-Bayona & Comstock, 2019). Cloning was carried out using *Escherichia coli* PIR1 cells (ThermoFischer Scientific). The plasmids were transferred into *B. thetaiotaomicron* VPI-5482 using triparental mating with *E. coli* PIR1 cells containing the pKO plasmid as the donor and *E. coli* pRK2013 (Clontech) as the helper strains. Cells were recovered on BHIH agar containing gentamicin (200 μg/ml) and erythromycin (25 μg/ml). Individual colonies were then grown overnight in BHIH containing gentamicin and erythromycin and streaked out on BHIH agar plates containing gentamicin and anhydrotetracycline (100 ng/ml). Individual colonies were then tested using PCR for the loss of the target gene.

2.8 | Salmonella bioassays

For the plate test assay, *Salmonella* AR2680 was grown on Luria broth-agar plates overnight at 37°C and streaked on *Salmonella* test media (47.8-mM Na_2HPO_4; 22-mM KH_2PO_4; 18.7-mM NH_4Cl; 8.6-mM NaCl; 21-mM Glucose; 2-mM $MgSO_4$; 0.1-mM $CaCl_2$) plate (15 g/L bacto-agar) supplemented with 335-μM *L*-methionine (Raux et al., 1996). After overnight growth at 37°C, the cells were scraped from the plate and washed three times with 154-mM NaCl. The plates were poured from washed cells mixed with 300 ml of *Salmonella* test media supplemented with bacto-agar at a temperature of 48°C. For mixed drops, 5 μl of 0.5-μM B_{12} or 17 mM *L*-methionine was mixed with 5 μl 10^{12} ml^{-1} BtBEVs and added to the plate. For exclusion drops, 5 μl of 0.5-μM B_{12} or 17-mM *L*-methionine was placed next to a 5-μl drop of 10^{12} ml^{-1} BtBEVs. The plates were then incubated overnight at 37°C and imaged. Replicates were not carried out for this experiment.

For the liquid test assay, *Salmonella* AR2680 was grown from a frozen stock for 6 h in Luria broth at 37°C with gentle agitation. One hundred microlitres were then sub-cultured into *Salmonella* test media and grown overnight to deplete the culture of vitamin B_{12}. Ten microlitres of depleted culture were used to inoculate 1 ml of *Salmonella* test media containing varying B_{12} and BtBEV concentrations in a 24-well tissue culture treated plate (Greiner) using three rows as triplicates and fourth row as a positive control. Sterile gas permeable adhesive seals (Fischer Scientific) were used to seal the plate to limit evaporation. The plates were incubated for 24 h at 37°C followed by OD_{600} readings.

2.9 | Fluorescent B_{12} synthesis

B_{12}-Cyanine 5 conjugate (fB12) was prepared from vitamin B_{12} in three steps (Figure S3) (1; B_{12}-succinate preparation based on Hannak et al.). One hundred milligrams vitamin B_{12} were resuspended in 4-ml DMSO, mixed with 30-mg succinic anhydride,

and heated at 75°C for 3 h (Hannak et al., 2007). The reaction was then precipitated by the addition of 40-ml ethyl acetate (EtOAc) and spun down at 2000 g for 5 min. The precipitate was then washed in EtOAc and recovered via centrifugation. The resulting red solid was dried at 50°C (yield = 86 mg; 80%) (2; B_{12}-succinate PFP ester preparation). Twenty-five milligrams of B_{12}-succinate were dissolved in 2.5-ml DMF followed by the addition of 75-μl triethylamine and 62.5-μl pentafluorophenyltrifluoroacetate. The reaction was incubated at 20°C for 30 min, precipitated by the addition of 25-ml EtOAc and spun down at 2000 g for 5 min. The precipitate was then washed in EtOAc and recovered via centrifugation. The resulting red solid was dried at 50°C (yield = 25 mg; 90%) and used without further purification (3; B_{12}-Cyanine 5(fB12) preparation). 2.98 mg B_{12}-succinate PFP ester was dissolved in 500-μl DMSO and treated with 1-μl triethylamine followed by the addition of 1.2-mg Cyanine 5 amine. The reaction was incubated at 20°C for 2 h and precipitated by the addition of diethyl ether (Et_2O). The resulting solid was isolated by centrifugation at 2000 *g* for 5 min. The precipitate was then washed in Et_2O and recovered via centrifugation. The resulting red solid was dried at 50°C (yield = 3 mg; 85%).

Intermediates and final product were analyzed using LC-MS using the Thermo MSQ plus system. The chromatography was carried out using a Phenomenex Luna C18 4.6 × 150-mm column at a flow rate of 1.5 ml/min, with a gradient of 98:2 (0.1% formic acid: methanol/0.1% formic acid) to 0:100 over the course of 7 min with a total LC run time of 13 min. Sample detection was carried out at 254 nm. The mass chromatograms were collected using electrospray ionization with a probe temperature of 500°C and voltage of 150 V.

2.10 | Fluorescent microscopy of Caco-2 cells

Twenty microlitres of 10^{11} ml-1 BtBEVs saturated with fB12 was mixed with 180-μl culture media (Prepared in 500 ml: 445-ml EMEM (EBSS) (Sigma M5650 contains 1% NEAA), 50 ml 10% FBS (BioSera FB-1001) and 5 ml 2-mM L-glutamine (Sigma G7513)) and added to Caco-2 monolayers cultured on collagen solution coated 24-well chamber slides followed by a 20-h incubation. Samples were fixed using 4% paraformaldehyde, permeabilized with 0.25% Triton X100 and blocked with 10% goat serum in PBS.

Immunolabeling was carried out for early endosomes (anti-Rab5(Abcam; ab18211)) or lysosomes (anti-LAMP1(Santa Cruz Biotechnology; sc-20011)) visualizing using an Alexa 488-conjugated secondary antibody (Invitrogen). Lyso-ID green detection kit (ENZO) was used to label lysozymes in live cells according to manufacturer's guidelines. For nuclear visualization, cells were stained with Hoechst 33342. Cells were mounted on high precision glass slides using Fluoromount-G antifade mounting medium.

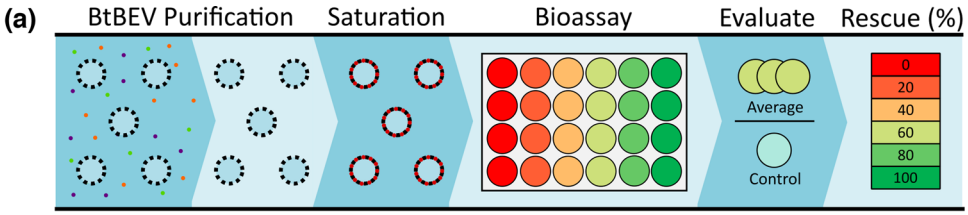
Imaging was carried using a Zeiss AxioImager M2 LSM880 Airyscan confocal microscope, equipped with a Plan-Apochromat 63×/1.4 oil or 20×/0.8 M27 (LysoID samples) objective operated via ZEN black software. Fluorescence was recorded at 405 nm (blue, nucleus), 488 nm (green, immunostaining) and 647 nm (red, fB12). To visualize intracellular signal, Z-stack images (4.4–8.8 μm) were obtained at 0.63 μm per slice. All image analysis was performed using Image J/FIJI v1.53c. Uptake of BtBEVs by Caco-2 was quantified using puncta counts per cell using an automated macro. Colocalization analysis of pixel intensity was carried out using the Colocalization Threshold Test plugin.

3 | RESULTS

3.1 | BtBEVs bind vitamin B_{12} via proteins involved in cellular uptake

The published *B. thetaiotaomicron* VPI-5482 bacterial extracellular vesicle (BtBEV) proteome contains the three recently described cobamide binding lipoproteins BtuG1 (Uniprot: Q8A7N4), BtuG2 (Q8A6D0) and BtuG3 (Q8A5Z1), which are encoded within the three unique cobamide uptake loci (Figure 1c) (Degnan et al., 2014; Valguarnera et al., 2018; Wexler et al., 2018). We postulated that if these binding proteins are located on the surface of BtBEVs, they may bind and deliver cobamides to the bacterium as part of a foraging system for essential nutrients. To test this hypothesis, *B. thetaiotaomicron* VPI-5482 were cultured in a rich media supplemented with *L*-methionine followed by starvation using a defined media lacking cobamides or *L*-methionine (Figure 2a). Starved cells were then used to inoculate test conditions, grown to stationary phase, quantified and compared to a control strain to which a saturating level of *L*-methionine had been added.

BtBEVs purified from media containing vitamin B_{12} rescued the growth of *B. thetaiotaomicron* cells (Figure 2b). By contrast, vesicles prepared from media containing the essential amino acid *L*-methionine instead of cobamide were unable to rescue growth. To determine the vitamin B_{12} binding capacity of BtBEVs, vesicles isolated from cultures containing either vitamin B_{12} or *L*-methionine were saturated with cobalamin and washed multiple times to remove unbound material. These vesicles were then analyzed using the growth assay and compared to samples containing standard concentrations of vitamin B_{12}. The B_{12} binding capacity of BtBEVs produced in media containing vitamin B_{12} was estimated at 1.3 ± 0.17 pmol/1010 vesicles, or approximately 78 molecules of B_{12} per vesicle. Interestingly, BtBEVs isolated from cultures containing *L*-methionine instead of cobalamin had a similar binding capacity (1.9 ± 0.30 pmol/10^{10} bacterial extracellular vesicles [BEVs]), consistent with the regulation of vitamin B_{12} binding activity being independent of the concentration of cobalamin used in the growth media.



(b)

Strain	BtBEV	
	WT_B12	WT_Met
VPI-5482 (WT)	62±7	2±1

(c)

Strain	BtBEV-B12	
	WT	Δ123
VPI-5482 (WT)	86±5	0±0
VPI-5482 Δtdk Δlocus1 Δlocus2 Δlocus3 (Δ123)	0±0	0±0

(d)

Strain	BtBEV-Corrinoid				
	5MB	FIII	FIIIM	Ps	FA
VPI-5482 (WT)	110±1	74±2	115±2	109±1	114±1

FIGURE 2 *B. thetaiotaomicron* growth rescue bioassay demonstrating BtBEV binding and delivery of a range of different cobamides. (a) Schematic of the assay workflow. Purified BtBEVs were saturated with B_{12} and used to recover cobamide starved *B. thetaiotaomicron* VPI-5482 or mutant strains. Assays were carried out in 24-well microtiter plates in triplicate using 10^{10} ml-1 BtBEVs. Rescue (%) represents the percentage of growth recovered compared to a positive control culture grown using 200 μM *L*-methionine. The boxed data represent the averaged triplicate and are colored according to the heatmap scale indicated. (b) Rescue of wild-type (WT) *B. thetaiotaomicron* VPI-5482 using BtBEVs derived from a culture containing either vitamin B_{12} (WT_B12) or *L*-methionine (WT_Met). Vitamin B_{12} saturation of the BtBEVs was not carried out for this experiment. (c) Rescue of WT or Δ*locus1* Δ*locus2* Δ*locus3* (Δ123) *B. thetaiotaomicron* VPI-5482 using B_{12} saturated BtBEVs derived from these strains grown in vitamin B_{12} free media. Unsaturated and saturation process controls showed no growth and are outlined in Figure S1. (d) Rescue of WT *B. thetaiotaomicron* VPI-5482 using WT BtBEVs produced in vitamin B_{12} free media and saturated with various cobamides (Figure 1; FIII, Factor III; FIIIM, Factor IIIM; Ps, Pseudovitamin B_{12}; FA, Factor A). BtBEV only controls showed no growth as observed previously (Figure S1).

The contribution of *B. thetaiotaomicron* cobamide uptake loci in the BtBEV cobalamin binding and uptake activity was determined using a cobamide uptake mutant lacking all three cobamide uptake loci (VPI-5482 Δ*locus1* Δ*locus2* Δ*locus3*; Δ123) (Degnan et al., 2014; Wexler et al., 2018). We compared BtBEVs produced by this mutant strain to wild-type (WT) BtBEVs. The vesicles were prepared from relevant cultures containing *L*-methionine but no cobamides, saturated with vitamin B_{12} and then analyzed using our rescue bioassay with WT and Δ123 strains (Figures 2c and S1). As expected, BtBEVs produced from the Δ123 mutant strain were unable to rescue the growth of WT *B. thetaiotaomicron* while WT BtBEVs promoted growth. This demonstrates that the BtBEV cobamide binding activity is encoded within the three cobamide uptake loci. Furthermore, the Δ123 strain was not rescued by WT BtBEVs loaded with vitamin B_{12} confirming the requirement of the uptake machinery within the cell, and not just its presence on BtBEVs.

3.2 | BtBEVs bind and deliver naturally occurring B_{12} analogues to *B. thetaiotaomicron*

B. thetaiotaomicron utilizes a variety of cobamides via proteins encoded by the three different cobamide uptake loci, which, as we have shown, are responsible for vitamin B_{12} binding to BtBEVs (Degnan et al., 2014). We, therefore, postulated that BtBEVs bind a range of cobamides. To test this hypothesis, five different cobamides (Figure 1b) were evaluated in our rescue bioassay (Figure 2a) using WT BtBEVs. The cobamides Coβ-cyano-(5-methylbenzimidazolyl)-cobamide (5 MB); Coβ-cyano-(5-hydroxybenzimidazolyl)-cobamide (Factor III); Coβ-cyano-(5-methoxybenzimidazolyl)-cobamide (Factor IIIM); Coβ-cyano-adeninyl-cobamide (Pseudovitamin B_{12}) and Coβ-cyano-(2-methyladeninyl)-cobamide (Factor A) (Figure 1b) were all found to bind BtBEVs and rescue the growth of *B. thetaiotaomicron* VPI-5482 cells (Figure 2d). Notably, the 5-hydroxybenzimidazolyl-cobamide was less efficient in comparison to the other cobalamin analogues, which may reflect this cobamide being less catalytically efficient as a cofactor for methionine synthase or being less efficiently bound and taken up by the bacterium.

3.3 | BtBEVs sequester vitamin B_{12} from Salmonella

Based on our findings and the competitive environment of the mammalian gut, we postulated that BtBEVs sequester cobamides from other potential users. This hypothesis was tested using a *Salmonella enterica* (Δ*metE* Δ*cbiB*) vitamin B_{12} auxotroph strain as

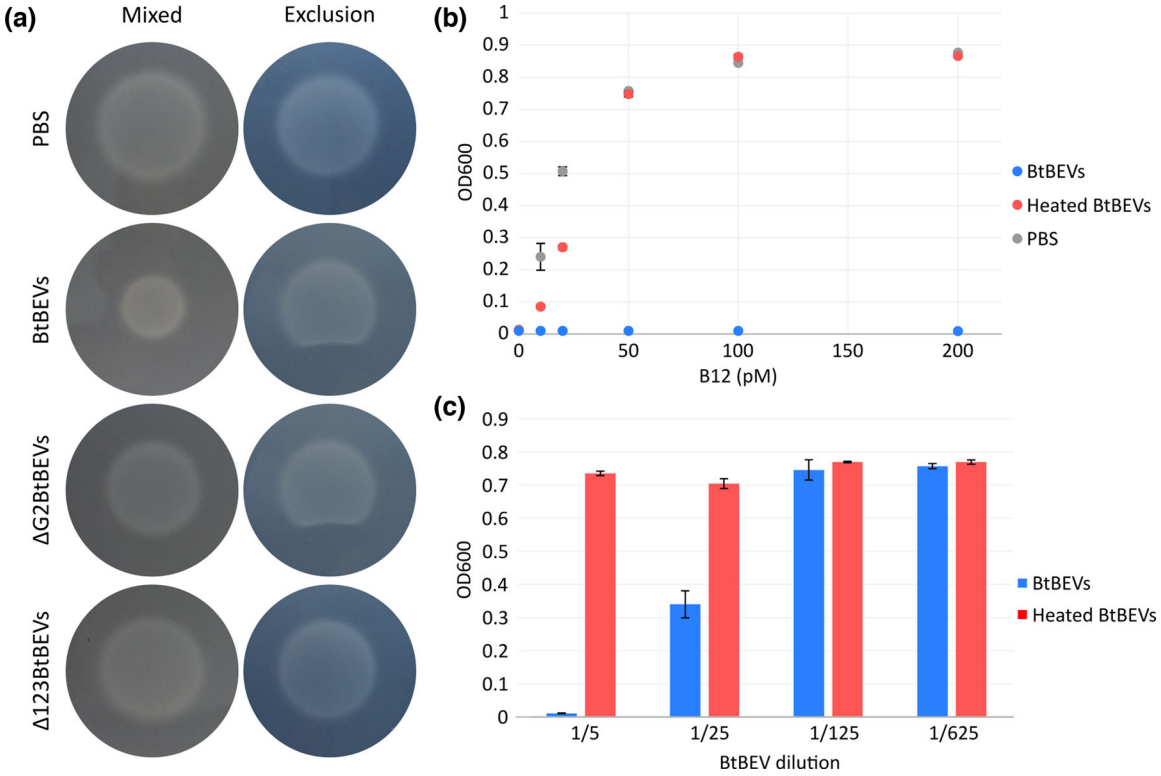


FIGURE 3 *Salmonella enterica* bioassays showing BtBEV inhibition of vitamin B_{12}-dependent growth. (a) Diffusion-based *S. enterica* bioassay plates showing reduction in vitamin B_{12}-dependent growth when BtBEVs are present. Inhibition of growth is observed when BtBEVs and, to a lower extent, ΔG2BtBEVs are tested. Complete loss of inhibition is observed when the triple mutant (Δ123BtBEVs) vesicles are used. Mixed: BtBEVs pre-mixed with vitamin B_{12} placed on the plate; reduction in colony size indicates inhibition. Exclusion: BtBEV and vitamin B_{12} drops placed adjacent to each other; exclusion of growth indicates inhibition. PBS, buffer control; ΔG2BtBEVs, BtBEVs from VPI-5482 Δ*btuG2*; Δ123BtBEVs, BtBEVs from VPI-5482 Δ*locus1* Δ*locus2* Δ*locus3*. Additional controls are shown in Figure S2. (b) Liquid culture *S. enterica* bioassay showing complete inhibition of growth by 10^{10} ml-1 BtBEVs at B_{12} concentrations up to 200 pM. This activity is lost when heat-treated vesicles are used. Experiment carried out in triplicate with error bars showing one standard deviation. (c) 10^{10} ml-1 BtBEV dilutions tested using liquid culture *S. enterica* bioassay shows the concentration dependence of growth inhibition. This activity is lost when heat-treated vesicles are used. Experiment carried out in triplicate with error bars showing one standard deviation.

an indicator strain in an agar diffusion plate assay to detect the presence of cobamides, where growth is dependent on the availability of cobamide (Raux et al., 1996). Using this positive growth bioassay plate method, the *S. enterica* auxotroph growth circle was reduced when vitamin B_{12} is delivered together with WT BtBEVs (Figure 3a). Furthermore, when *L*-methionine was used instead of the vitamin or the vesicles were boiled, no growth inhibition was observed demonstrating that the BtBEVs sequester vitamin B_{12} from *S. enterica* (Figure S2). Pre-incubation of vitamin B_{12} with BtBEVs was not required as demonstrated by the exclusion of growth when a drop containing BtBEVs was placed adjacent to the vitamin B_{12} drop, a method developed to investigate the inhibitory action of metal-substituted cobalamins (Figures 3a and S2) (Widner et al., 2016). We postulated that BtuG2, due to its high affinity for cobamides, is responsible for the observed inhibition (Wexler et al., 2018). We investigated this by comparing BtBEVs produced by a novel VPI-5482 Δ*btuG2*(ΔG2BtBEVs) mutant strain to BtBEVs obtained from a strain deficient in all three vitamin B_{12} uptake loci (Δ123BtBEVs) (Figures 3a and S2). ΔG2BtBEVs appeared to inhibit the growth of *S. enterica* to a lesser extent than the WT vesicles while the Δ123BtBEVs showed no clear inhibition. This supports the hypothesis that BtuG2 is involved in this inhibitory activity with other proteins in the loci also playing a role, however, further experimentation is required to confirm and quantify these observations. Overall, BtBEVs inhibit the growth of vitamin B_{12} auxotrophs and can therefore act as antimicrobial agents through limiting nutrient bioavailability.

The effect of BtBEVs on vitamin B_{12} utilization in *S. enterica* was confirmed in liquid culture (Figure 3b). WT BtBEVs prevented growth at all tested vitamin B_{12} concentrations whereas heat-inactivated BtBEVs showed no inhibitory effects. When *L*-methionine was used instead of B_{12}, no inhibition of growth was observed (BtBEV: 0.947 ± 0.008; Heated BtBEV: 0.958 ± 0.003). To estimate the binding capacity of BtBEVs, a range of BtBEV dilutions was assessed using a constant vitamin B_{12} concentration (Figure 3c). The binding capacity of BtBEVs was estimated to be around 0.3375 pmol vitamin B_{12} per 10^{10} BtBEV, equating to around 20 molecules of vitamin B_{12} per BtBEV, lower than the binding capacity of 78 molecules estimated using the *Bacteroides* rescue assay. This variation may be explained by the limitations in the accurate quantification of the BtBEV concentration or by different vitamin B_{12} affinities of the binding proteins. For instance, *S. enterica* may utilize cobalamin bound to BtuG1 or BtuG3 if these proteins have a lower affinity for the nutrient, which would reduce the overall binding capacity estimated using this method.

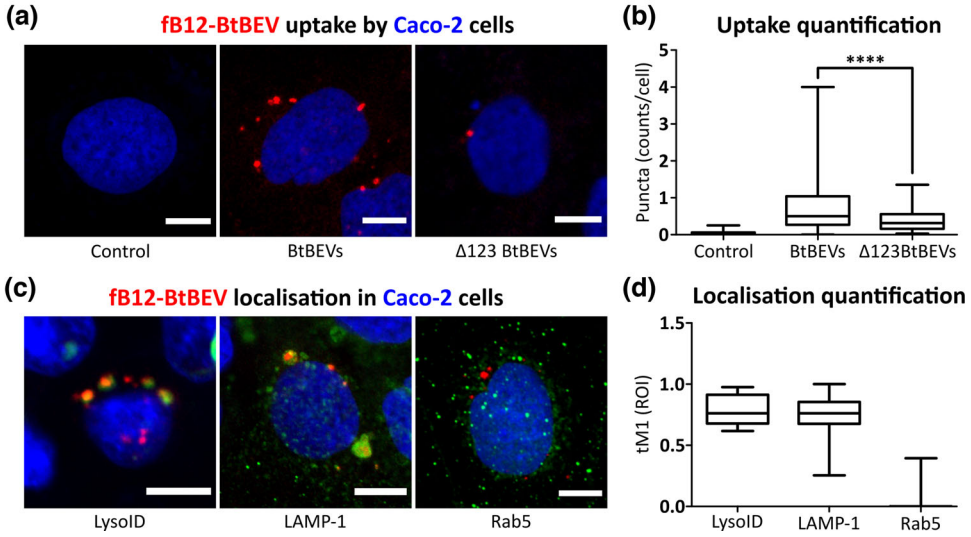


FIGURE 4 Confocal fluorescence microscopy experiments showing the uptake and localization of BtBEV saturated with fB12 using Caco-2 human intestinal epithelial cells. (a) Confocal fluorescence microscopy images showing the uptake of fluorescent B_{12} (fB12; red) bound to WT or mutant (Δ*locus1* Δ*locus2* Δ*locus3*; Δ123) BtBEVs by human intestinal epithelial cells (Caco-2; blue nuclear stain) cells. Control cells contain BtBEVs with no bound fB12. Scale bars: 10 μm. Additional representative images are available as Figure S4. (b) Quantification of fB12 uptake referring to samples shown in part (a) of the figure. Additional control counts were similar to the buffer only control shown and are available in Figure S5. (c) Confocal fluorescence microscopy images showing the localization of fB12 (red) delivered via BtBEVs to lysosomes (LysoID and LAMP-1; green) but not early endosomes (Rab5; green) within Caco-2 cells (blue nuclear stain). Scale bars: 10 μm. Cross sections of LAMP-1 and Rab5 (Figure S6) and additional representative images for all samples (Figure S7) are available in Supplementary information. (d) Quantitative analysis of fB12 co-localization with lysosomes (LysoID and LAMP-1) or early endosomes (Rab5) using thresholded Mander's split colocalization coefficients, where 1 is indicative of perfect co-localization and 0 with no co-localization. Significant co-localization observed for LysoID and LAMP-1, but not Rab5 labelling.

The ability of BtBEVs to suppress the growth of other bacteria via exogenous vitamin B_{12} sequestration has important implications for gut microbiome makeup, with the ability of bacteria expressing high-affinity cobamide-binding proteins suppressing growth and providing a competitive growth advantage over other bacterial cobamide auxotrophs (Bauer et al., 2018; Degnan et al., 2014; Magnúsdóttir et al., 2015). Furthermore, as cobamides play an essential role in ethanolamine and propanediol utilization by a variety of pathogens their sequestration might limit the ability of certain pathogens to colonize the gut (Anast et al., 2020; Faber et al., 2017; Garsin, 2010).

3.4 | BtBEVs deliver vitamin B_{12} to human intestinal epithelial cells

Previously, we have shown that BtBEVs interact with and are acquired by intestinal epithelial cells both *in vitro* and *in vivo* (Jones et al., 2020). The binding of cobamides to BtBEVs would permit a previously unrecognized route for their acquisition. To demonstrate that BtBEVs can mediate cobamide transport into epithelial cells, vitamin B_{12} was chemically labelled by attachment of a cyanine 5 fluorescent dye to the 5′-OH of the ribose group of the lower nucleotide loop (fB12; Figure S3), prior to incubation with BtBEVs produced from *B. thetaiotaomicron* VPI-5482 WT (BtBEVs) or *B. thetaiotaomicron* VPI-5482 Δ*locus1* Δ*locus2* Δ*locus3* (Δ123BtBEVs) cells in cobamide free media. fB12-labelled vesicles and appropriate controls were then incubated with Caco-2 human intestinal epithelial cells and stained with the nuclear Hoechst 33342 stain. Confocal fluorescent microscopy (Figures 4a and S4) was used to observe and quantify uptake of fB12-BtBEVs (Figures 4b and S5). A significant reduction in fluorescence signal was observed when Δ123BtBEVs were used. Using LAMP-1 antibodies and the LysoID acidic organelle selective dye, internalized fB12 was shown to localize to lysosomes (Figures 4c, 4d, S6 and S7). By contrast, fB12 signal was not associated with early endosomes as demonstrated by no co-localization with the early endosome marker Rab5 (Figures 4c, d and S7).

Significantly, lysosomal trafficking is the natural uptake route for vitamin B_{12}, which is delivered via the highly specific intrinsic factor protein and exported via a specialized transporter (Kozyraki et al., 1999; Smith et al., 2018). As the vesicles bind other cobamides and deliver cargo to the lysosomes, it is possible, depending on the specificity of the lysosomal transporter, that these analogues are then trafficked into and throughout the host organism. An analysis of the amino acid sequence of BtuG2 with the program Procleave (Li et al., 2020), which predicts potential proteolytic sites, highlights multiple points within the primary structure where the protein would likely be degraded by cathepsin D, a ubiquitously expressed lysosomal protease. Hence it is

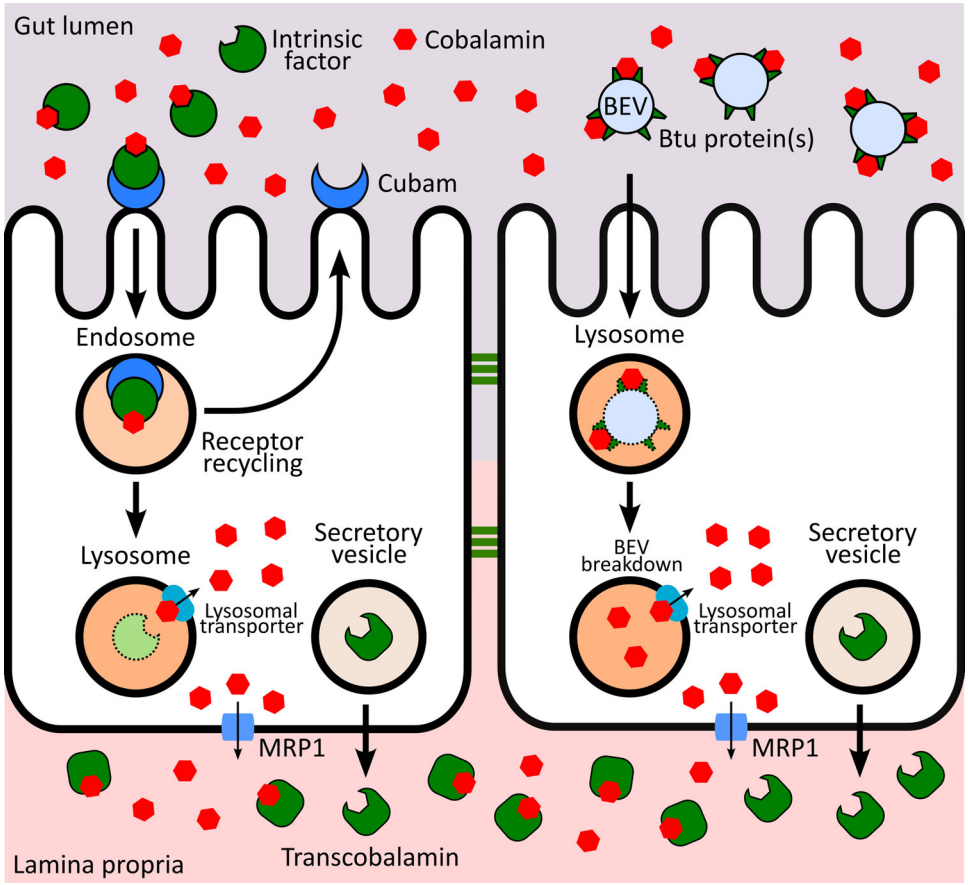


FIGURE 5 Proposed model of cobalamin uptake via BEVs and its similarities to intrinsic factor-dependent uptake. Left image depicts the mechanism of intrinsic factor-dependent uptake. Intrinsic factor binds cobalamin in the gut lumen and is internalised by barrier epithelial cells via binding to the cubam receptor complex. The bound complex is delivered to endosomes where the receptor complex is recycled while the cobalamin bound intrinsic factor is transported into lysosomes leading to its degradation and the release of cobalamin. Lysosomal cobalamin is then taken up into the cell where it is utilised and exported into the underlying lamina propria via the multidrug resistance protein 1 (MRP1) transporter. The cells also secrete transcobalamin which binds cobalamin and delivers it to other cells in the body. Right image shows the proposed mechanism for BEV-mediated cobalamin delivery. BEVs bound to cobalamin are acquired by epithelial cells and taken up into lysosomes where they are degraded leading to the release of bound cobalamin which can then be disseminated in an identical manner.

very likely that BEV-bound B_{12} would be released within the lysosome for further transport. The proposed model of cobalamin uptake via BEVs and its similarities to intrinsic factor-mediated uptake is compared in Figure 5.

4 | DISCUSSION

In this work, we have shown that BtBEVs can bind and deliver a range of cobamides to *B. thetaiotaomicron* in a gene-dependent manner while sequestering these from competing organisms. The binding of cobamides to BtBEVs is mediated by the presence of the BtuG proteins, a seven-bladed β-propeller vitamin B_{12}-binding protein family that appears to be restricted to *Bacteroidetes* (Wexler et al., 2018). Due to their high-binding affinity and presence in BEVs, the system represents a novel mechanism for limiting growth of cobamide-dependent competitors as demonstrated here using a mutant *S. enterica* cobalamin auxotroph strain. Both micronutrient scavenging and sequestration constitute novel functions for BEVs, which may not be limited to cobamides as, for instance, proteins associated with iron acquisition are also present within the BtBEV proteome (Valguarnera et al., 2018). The inclusion of other proteins and enzymes such as cephalosporinases in BtBEVs suggest that they play a wider role in the maintenance of a balanced microbiota (Stentz et al., 2015). The contribution that BEVs make to intra- and inter-kingdom interactions exemplified here raises several interesting and important questions that require additional research including how BEV production and cargo selection are regulated, and whether host factors contribute to this process and, if so, which ones and how?

It is also important to note the limitations of the experiments, especially around the production and accurate quantification of BEVs. For our experiments, we produced vesicles in a defined, particle-free media. We note that a large proportion of currently

published papers around the topic of extracellular vesicles relies on complex media containing liposomes and other nanoparticles that not only influence the quantification of the particles but will likely influence any downstream experimentation. Regarding quantification, we relied solely on particle tracking analysis using the ZetaView instrument. However, even when using defined media, we noted both yield and size variation, both of which may contribute to the accuracy of quantification thereby affecting any downstream results. We, therefore, note that any quantitative data provided in the paper should be interpreted with care and is likely to be adjusted as better production and quantification methods are developed.

Our finding that BtBEVs can bind different cobalamin analogues and transport them to model gut epithelium cells represents a potential previously unrecognized route for their entry into the circulatory system, providing an explanation for their presence in human serum and organs (Kanazawa & Herbert, 1983) (Figure 5; Kolhouse et al., 1978). Despite bacteria within the large intestine producing an extensive range of non-cobalamin cobamides, they have largely been ignored as a potential source of vitamin B_{12} analogues since they are present downstream of the ileum, the main cobalamin absorption site (Allen & Stabler, 2008; Donaldson et al., 2016). It has been demonstrated that human cobalamin-dependent enzymes can function *in vitro* using some of these analogues, but animal studies suggest that these are not functional *in vivo* (Kolhouse et al., 1991; Sokolovskaya et al., 2021; Stabler et al., 1991). Furthermore, the presence of analogues in patients with low cobalamin levels has been associated with human neurological abnormalities and hence there is a need to revisit the presence and effect of vitamin B_{12} analogues on human health (Carmel et al., 1988)

ACKNOWLEDGEMENT

We are grateful to Prof Andrew Goodman (Yale) for providing the triple mutant *Bacteroides thetaiotaomicron* VPI-5482 (Δ123) strain and comments on the manuscript. This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) under Grant BB/R012490/1; BBSRC under Grant BBS/E/F/000PR10353; BBSRC under Grant BBS/E/F/000PR10355; BBSRC under Grant BBS/E/F/000PR10346; BBSRC under Grant BB/S002197/1 and Royal Society under Grant INF\R2\180062. This project was performed in Norwich and Canterbury in United Kingdom.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

Data supporting the findings of this study are available within the article and its supplementary materials. Strains are available from the corresponding authors.

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How to cite this article: Juodeikis, R., Jones, E., Deery, E., Beal, D. M., Stentz, R., Kräutler, B., Carding, S. R., & Warren, M. J. (2022). Nutrient smuggling: Commensal gut bacteria-derived extracellular vesicles scavenge vitamin B12 and related cobamides for microbe and host acquisition. *Journal of Extracellular Biology*, *1,* e61. https://doi.org/10.1002/jex2.61