RESEARCH ARTICLE

A Phytase-Based Reporter System for Identification of Functional Secretion Signals in Bifidobacteria

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Abstract

Health-promoting effects have been attributed to a number of Bifidobacterium sp. strains. These effects as well as the ability to colonise the host depend on secreted proteins. Moreover, rational design of protein secretion systems bears the potential for the generation of novel probiotic bifidobacteria with improved health-promoting or therapeutic properties. To date, there is only very limited data on secretion signals of bifidobacteria available. Using in silico analysis, we demonstrate that all bifidobacteria encode the major components of Sec-dependent secretion machineries but only B. longum strains harbour Tat protein translocation systems. A reporter plasmid for secretion signals in bifidobacteria was established by fusing the coding sequence of the signal peptide of a sialidase of Bifidobacterium bifidum S17 to the phytase gene appA of E. coli. The recombinant strain showed increased phytase activity in spent culture supernatants and reduced phytase levels in crude extracts compared to the control indicating efficient phytase secretion. The reporter plasmid was used to screen seven predicted signal peptides in B. bifidum S17 and B. longum E18. The tested signal peptides differed substantially in their efficacy to mediate protein secretion in different host strains. An efficient signal peptide was used for expression and secretion of a therapeutically relevant protein in B. bifidum S17. Expression of a secreted cytosine deaminase led to a 100-fold reduced sensitivity of B. bifidum S17 to 5-fluorocytosine compared to the non-secreted cytosine deaminase suggesting efficient conversion of 5-fluorocytosine to the cytotoxic cancer drug 5-fluorouracil by cytosine deaminase occurred outside the bacterial cell. Selection of appropriate signal peptides for defined protein secretion might improve therapeutic efficacy as well as probiotic properties of bifidobacteria.

Introduction

Bifidobacteria are an important component of the normal human gastrointestinal microbiota and, besides lactobacilli, the most frequently used microbial supplements in functional foods and probiotic formulations [1]. The concept of functional foods containing live microbial
supplements, i.e. probiotics, is based on the observation that some of the commensal bacteria of the human microbiota have beneficial effects in different in vitro settings, small animal models or clinical trials [1,2]. These beneficial effects are, in most cases, strain-specific and include maintenance of remission in paediatric ulcerative colitis (UC), prevention of *Clostridium difficile* - and antibiotic-associated diarrhoea, and a reduced mortality in necrotizing enterocolitis (NEC) [2].

Some of the health-promoting effects of probiotics and bifidobacteria seem to be mediated by secreted factors. Several anti-inflammatory factors of bifidobacteria potentially involved in their effects in UC and NEC are secreted proteins. One example is a eukaryotic-type serine protease inhibitor (serpin). Serpin was initially identified in the genome sequence of *B. longum* NCC2705 [3] and shown to inhibit pancreatic and neutrophil elastases [4]. Later, serpin-like proteases were also identified in other bifidobacteria [5,6]. Serpin is hypothesized to confer protection against proteolysis by pancreatic elastase in the gastrointestinal environment as well as to inhibit proteolytic damage by neutrophil elastase during intestinal inflammation [4]. A secreted protein of *B. animalis* subsp. *lactis* was shown to inhibit inflammatory chemokine secretion by TNF-α challenged cultured intestinal epithelial cells. Similarly, a released peptide factor of *B. infantis* was able to prevent loss of epithelial integrity in response to TNF-α or IFN-γ challenge in an in vitro setting [7].

The ability to exert a health-promoting effect via secreted proteins depends on an active metabolism and, thus, on acquisition of nutrients. Various strains and species of bifidobacteria were shown to ferment different high molecular weight substrates derived from both the host and its diet including mucus [8], human milk oligosaccharides [9,10], starch [11], and other plant-derived polysaccharides [12]. All these substrates require degradation by extracellular enzymes prior to uptake and further breakdown for energy conservation. The ability to utilize these substrates is thought to provide a selective advantage over other bacteria, aiding the colonization of breast-fed infants and persistence during later stages of life [13–16] and hence support their health-promoting effects.

In addition to their reported health-promoting effects, some strains of bifidobacteria were shown colonise solid tumours in various mouse models [17–20] and thus have gained increasing interest as vectors for delivery of therapeutic genes in cancer therapy [21–23]. The most widely used approach in bacterial tumour targeting is expression of enzymes that convert non-toxic prodrugs to therapeutically active compounds [24]. One example of a well-studied prodrug-converting enzyme (PCE) is cytosine deaminase (CD). This enzyme converts the non-toxic prodrug 5-fluorocytosine to 5-fluorouracil, which interferes with DNA synthesis and thus inhibits proliferation of tumour cells. In order to improve the efficacy of prodrug conversion, while at the same time avoiding inhibitory effects on the proliferation of the delivery vector, PCEs are mostly used as secreted proteins.

Altogether, these findings indicate that secreted proteins might be important for establishment and maintenance of stable bifidobacterial populations in the gastrointestinal tract. Moreover, efficient protein secretion is crucial for functionality of bifidobacteria as probiotics and gene delivery vectors for tumour targeting approaches. In bacteria, the majority of extracellular proteins is secreted by either the Sec or the Twin arginine translocation (Tat) pathway [25,26]. The Sec pathway exports proteins across the cytoplasm membrane in an unfolded state, whereas the Tat-pathway transports folded proteins [27,28]. Both pathways depend on secretion signals usually located in the N-terminus of the substrate that are distinct yet share structural similarities [27].

In bifidobacteria, protein secretion has not been analysed in great detail and there is only one study employing a nuclease reporter to identify bifidobacterial signal sequences [29]. In the present study, we aim at providing a more systematic analysis of protein secretion and
associated signal peptides of bifidobacteria, developing a system to analyse these SPs, and
devising a tool for efficient expression of extracellular proteins in bifidobacteria.

**Materials and Methods**

**Bacterial strains, plasmids and growth conditions**

All strains and plasmids used in this study are listed in S1 Table. *E. coli* DH10B was used as
cloning host and for propagation of plasmids and grown in Luria broth (LB) at 37°C. Bifido-
bacteria were grown anaerobically at 37°C in Reinforced Clostridial Medium (RCM, BD Difco,
Germany) or Lactobacilli MRS (BD Difco, Germany) broth supplemented with 0.5 g/L L-cyste-
ine hydrochloride-monohydrate (MRSc). For cultivation of *E. coli* and *Bifidobacterium sp.*
strains harbouring plasmids, 100 μg/ml spectinomycin were added to culture media. All media
were prepared with ultrapure water.

**Cloning procedures**

Genomic DNA of *E. coli* DH10B was used as template for amplification of *appA*. Coding
sequences of predicted SPs were amplified from genomic DNA of *B. bifidum* S17 or *B. longum*
E18. PCRs were performed using Phusion DNA Polymerase (Thermo Scientific, Germany). All
primers used in this study (S2 Table) were purchased from Eurofins Genomics GmbH (Ger-
many). Thermo cycling was performed on a FlexCycler (Analytik Jena, Germany) with anneal-
ing temperature optimized for each primer pair. The *appA* gene of *E. coli* K-12 encoding a
phytase was amplified without its native signal peptide sequence using primers PhytF and
PhytR. The obtained PCR product was digested with restriction enzymes *XhoI* and *HindIII*
and ligated to the 4,423 bp fragment of similarly digested pMDY23-P*gap* [30], i.e. the vector
backbone including P*gap* but lacking the gusA gene. This yielded pMgapP, which harbours the
*appA* gene fused directly to P*gap* without any signal sequence.

Coding sequences of signal peptides were fused to the *appA* gene by splicing-by-overlap-
extension (SOEing) PCR [31]. The coding sequences for different SPs were amplified using a
forward primer and a SOEing reverse primer. The PCR was designed to include two additional
amino acid residues after the predicted cleavage site to preserve the recognition sequence for
cleavage. In parallel, a SOEing forward primer with complementary sequence to the SP reverse
primer was used together with primer PhytR for amplification of *appA*. To fuse the SP coding
sequence to *appA*, a second round of PCR was performed using the two PCR products of the
first round as template, the SP forward primer, and primer PhytR. To increase specificity,
DMSO was added to the PCR reaction to a final concentration of 5% (v/v) and annealing tem-
perature was set to 70°C. The obtained PCR products were digested with restriction enzymes
*XhoI* and *HindIII* and ligated to the 4,423 bp fragment of *XhoI/HindIII* cut pMDY23-P*gap*
yielding plasmids with exact translational fusions of the different SPs to *AppA*.

In order to generate a vector for expression of a secreted cytosine deaminase (CD), the sig-
nal peptide of the *bbif_1734* gene encoding a sialidase was amplified from *B. bifidum* S17 chromo-
somal DNA by PCR using primers SP*fw_Sall* and SP*rev_HindIII*. The *codA* gene was
amplified from *E. coli* K-12 chromosomal DNA with the primers *codA_fw_HindIII* and
*codA_rev_SacI* by PCR. Both PCR products were digested with *HindIII* and subsequently
joined by a ligase reaction. The fusion was then amplified by PCR using primers *SP_fw_Sall*
*codA_rev_SacI* and the product was digested with *Sall* and *SacI* and ligated to the 4,423 bp
fragment of *Sall/SacI* cut pMGS-P*gap*-bopAHis6 [32] containing P*gap* to yield pAO-S0-CD.
The control plasmid pAO-CD, which contains a SP-less CD construct, was obtained by amplifi-
ying *codA* with primers *codA_fw_Sall* and *codA_rev_HindIII* and ligation of the *Sall/HindIII*
digested PCR product to to the 4,423 bp fragment of *Sall/HindIII* cut pMGS-P*gap*-bopAHis6.
Following transformation into *E. coli*, plasmids of spectinomycin resistant colonies were checked for correct inserts by PCR. Plasmids of positive clones were verified by restriction analysis and Sanger sequencing and constructs with correct sequences were transformed into either *B. bifidum* S17 or *B. longum* E18 as described elsewhere [33].

**Phytase assay**

For phytase samples, special attention is needed to deplete potential free phosphate contamination. All related reagents and medium were prepared in ultrapure ddH2O (18 MΩ·cm; Millipore, USA).

Recombinant *B. bifidum* S17 or *B. longum* E18 strains were grown in 50 ml RCM containing 100 μg/ml spectinomycin under anaerobic conditions. At the indicated time points, 5 ml of the cultures were harvested and centrifuged (5 000 × g, 5 min, 4°C). Supernatants were filter-sterilized and used for determination of extracellular phytase activity. Bacterial pellets were washed twice in 1 ml of 0.2 M sodium citrate buffer, pH 5.5, and resuspended in 500 μl of the same buffer. Bacterial suspensions were transferred to cryotubes containing 250 μg glass beads and disrupted during 2 cycles of 35 s at 6500 rpm in a Precellys 24 homogenisator (PEQLAB Biotechnologie GmbH, Germany). Lysates were centrifuged (13 000 × g, 5 min, 4°C) and the supernatant was retained as crude extracts for determination of intracellular phytase activity. Total protein in crude extracts was quantified using the Pierce BCA protein assay kit (Thermo Scientific, Germany).

Phytase activity in supernatants and crude extracts was quantified using an assay described elsewhere [34] with minor modifications. Briefly, 100 μl sample (supernatant or crude extract) were pre-incubated for 5 min at 37°C and then mixed with 100 μl 10.8 mM sodium phytate (50% (w/w) phytic acid diluted in 0.2 M sodium citrate buffer). The reaction was carried out at 37°C for 15 min, and then stopped by adding 200 μl 15% trichloroacetic acid (TCA). After centrifugation (14 000 × g, 2 min), an aliquot of 20 μl was mixed with 480 μl ultrapure ddH2O and 500 μl color reagent (mix of 1M sulfuric acid, 2.5% (w/v) ammonium molybdate in ddH2O, and 10% (w/v) ascorbic acid in ddH2O at a ratio of 3:1:1). The mixture was incubated at 50°C for 15 min, and 100 μl were transferred to a transparent 96-well microtiter plate (Thermo Scientific, Germany). Absorbance at 820 nm was measured in triplicate using an Infinite M200 multimode microplate reader (Tecan, Switzerland). Phytase activity equivalents were calculated using a standard curve of two-fold serial dilutions of a 9 mM potassium dihydrogen phosphate in water. Phytase activity equivalents were defined as the amount of enzyme that catalyses the release of 1 μmol of inorganic phosphate per minute from 5.4 mM sodium phytate (i.e. 100 μl sample + 100 μl 10.8 mM sodium phytate solution, see above) under the conditions of the assay. Activities were expressed as relative phytase units (RPU) per ml in supernatant and RPU/mg in crude extracts.

**Phytate degradation assays**

To test for phytate degradation an agar plate assay developed for yeasts [35] was adapted for bifidobacteria using RCA agar (RCA) containing calcium phytate (Ca-phytate) as substrate. RCA phytate agar was prepared by adding 3 g/l calcium carbonate (Sigma, Germany) and 1.5 ml of a 50% (w/w) phytic acid solution in H2O (Sigma) to standard RCA agar prior to autoclaving. 2 μl of an overnight culture of the tested recombinant bifidobacteria were spotted onto a freshly prepared agar plate. Following incubation for 48 h under standard conditions, phytate degradation can be observed by clear zones in the otherwise opaque agar around the bacterial spots on the plate.
Growth inhibition by 5-FC

The effect of the conversion of 5-FC to 5-FU by cytosine deaminase was determined for *B. bifidum* S17 wildtype and its isogenic derivatives carrying plasmids pAO-CD or pAO-S0_CD. OD₆₀₀ of overnight cultures grown in MRSc was adjusted to 0.1 in fresh medium containing 5-FC at 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005 or 0.001 mg/ml (final concentration). 200 μl aliquots were pipetted into wells of a 96-well microtiter plate in four technical replicates per strain and concentration. After 24 h of anaerobic incubation at 37°C, OD₆₀₀ was measured using an Infinite M200 multimode reader.

Bioinformatic analysis

The sequenced and annotated genomes of *B. longum* E18 (GenBank accession: CM002287), *B. bifidum* S17 (CP002220), *B. breve* S27 (CP006716), *B. animalis* subsp. *lactis* ATCC27673 (CP003941), *B. adolescentis* ATCC15703 (AP009256), and *B. dentium* Bd1 (CP001750) were searched for genes encoding Sec proteins. Homologies of the deduced amino acid sequences of bifidobacterial Sec homologues to the respective *E. coli* K12-W3110 proteins were calculated using the multiple alignment function of the Basic Local Alignment Search Tool (BLAST, www.ncbi.nlm.nih.gov/BLAST/).

*B. bifidum* S17 proteins with predicted extracellular localization were extracted from the precomputed genome results on the cPSORTdb database (version 3) [36]. The sequences of all proteins were analysed for potential SPs using SignalP Version 4.1 [37], TatP [38], and TAT-FIND [39].

Results

Analysis of protein secretion pathways in bifidobacteria

As a basis to establish a protein secretion reporter in bifidobacteria, the genome sequences of a number of representative *Bifidobacterium* sp. strains were analysed. As expected, all analysed genomes harboured genes for SecY, SecE, and SecG, i.e. the major components of the Sec translocon, and the associated ATPase SecA with reasonable homology to the respective proteins of *E. coli* K12-W3110 (Table 1). By contrast, genes for Tat-dependent protein secretion were only found in the genomes of *B. longum* E18 (Table 1) and other strains of this species (data not shown).

Construction of a secretion reporter for bifidobacteria

We next extracted a list of all proteins of *B. bifidum* S17 predicted to be localized to the extracellular compartment. The N-terminal 60 residues of all proteins retrieved were analysed in silico for potential SPs (Table 2). The SP of a sialidase (BBIF_1734) had the highest PSORTb E-score for extracellular localization and the second highest SignalP D-score for SP prediction. Moreover, the sialidase of *Micromonospora viridifaciens*, a closely related member of the phylum Actinobacteria, has been experimentally confirmed to be secreted into the extracellular environment [40,41]. Thus, the SP of BBIF_1734 was named S0 and selected to develop a secretion reporter using the phytase gene appA of *E. coli* DH10B.

The S0 sequence was fused to the appA gene by SOEing PCR and cloned into pMDY23-P_gap under the control of P_gap replacing the glucuronidase reporter gene gusA (Fig 1A) to yield pMgapS0P. As a control vector, pMgapP was constructed, which harbours an identical appA construct fused directly to P_gap without a signal sequence.

Both plasmids were transformed into *B. bifidum* S17 and phytase activity in crude extracts (intracellular) and culture supernatants (extracellular) of the recombinant strains was
measured at various time points during growth. Both strains displayed almost identical growth (data not shown) ruling out any effect of plasmids on growth or phytase activity. Phytase activity markedly increased over time in supernatants of \textit{B. bifidum} S17/pMgapS0P (Fig 1B). By contrast, phytase activity in supernatants of the control strain \textit{B. bifidum} S17/pMgapP were barely above background until later time points during growth, i.e. stationary growth phase (Fig 1B). On the other hand, phytase activities were higher in crude extracts of \textit{B. bifidum} S17/pMgapP than in the strain harbouring pMgapS0P, i.e. the construct with a SP, throughout the experiment (Fig 1C).

### Table 1. Components of the major protein secretion machineries encoded on the genomes of representative \textit{Bifidobacterium} sp.

| Species/component | Locus Tag | Size [aa]\(^a\) | Homology\(^b\) | GeneBank/RefSeq Accession |
|-------------------|-----------|----------------|--------------|---------------------------|
| \textit{B. longum} E18 |           |                |              |                           |
| SecY              | BLONG_1793 | 445            | 43%          | ESV34191.1                |
| SecE              | BLONG_2073 | 75             | 29%          | ESV34434.1                |
| SecG              | BLONG_1190 | 82             | 25%          | ESV36665.1                |
| SecA              | BLONG_1273 | 964            | 49%          | ESV33737.1                |
| TatA              | BLONG_0090 | 96             | 35%          | ESV32709.1                |
| TatB              | BLONG_0088 | 86             | 35%          | ESV32707.1                |
| TatC              | BLONG_0089 | 360            | 29%          | ESV32708.1                |
| \textit{B. bifidum} S17 |           |                |              |                           |
| SecY              | BBIF_1484  | 444            | 42%          | ADO53689.1                |
| SecE              | BBIF_0279  | 75             | 31%          | ADO52484.1                |
| SecG              | BBIF_0980  | 82             | 30%          | ADO53185.1                |
| SecA              | BBIF_1223  | 960            | 47%          | ADO53428.1                |
| \textit{B. breve} S27 |           |                |              |                           |
| SecY              | BS27_1602  | 445            | 43%          | AHJ25374.1                |
| SecE              | BS27_1723  | 75             | 29%          | AHJ25486.1                |
| SecG              | BS27_0998  | 82             | 25%          | AHJ24814.1                |
| SecA              | BS27_1200  | 960            | 46%          | AHJ24997.1                |
| \textit{B. animalis} \text{subsp.} \textit{lactis} ATCC27673 |           |                |              |                           |
| SecY              | BLAC_02015 | 449            | 42%          | AGW84625.1                |
| SecE              | BLAC_01550 | 76             | 30%          | AGW84532.1                |
| SecG              | BLAC_04320 | 82             | 31%          | AGW85057.1                |
| SecA              | BLAC_05590 | 974            | 45%          | AGW85303.1                |
| \textit{B. adolescens} ATCC15703 |           |                |              | NC_008618.1               |
| SecY              | BAD_0341   | 457            | 42%          | BAF39122.1                |
| SecE              | BAD_0245   | 75             | 30%          | BAF39026.1                |
| SecG              | BAD_0833   | 100            | 33%          | BAF39614.1                |
| SecA              | BAD_1020   | 958            | 46%          | BAF39801.1                |
| \textit{B. dentium} Bd1 |           |                |              |                           |
| SecY              | BDP_0451   | 457            | 42%          | ADB09126.1                |
| SecE              | BDP_0350   | 75             | 30%          | ADB09028.1                |
| SecG              | BDP_1140   | 82             | 33%          | ADB09771.1                |
| SecA              | BDP_1418   | 958            | 46%          | ADB10026.1                |

\(^a\)protein size in amino acid residues (aa).

\(^b\)percent identity on amino acid sequence level to the respective homologue of \textit{E. coli} K12-W3110.
Comparative analysis of various SPs in different *Bifidobacterium* sp. hosts

Following the successful establishment of *appA* as a secretion reporter in *B. bifidum* S17, this system was used to test various other bifidobacterial SPs. For this purpose, a total of six SPs with high D-scores according to the SignalP prediction were selected (Table 3). Of these SPs, two belong to proteins from *B. bifidum* S17 (BBIF_1681 and BBIF_1761) and two other to proteins from *B. longum* E18 (BLONG_1728 and BLONG_0476). Moreover, two SPs of (putative) Tat-secreted proteins of *B. longum* E18 (BLONG_0223 and BLONG_1620) were included. To test their functionality, all SPs were fused to the *appA* reporter by SOEing PCR, cloned into *pMDY23-P*gap replacing the *gusA* reporter gene, and the obtained plasmids were introduced into *B. bifidum* S17 or *B. longum* E18 by electroporation. Monitoring of growth in RCM broth indicated that all recombinant strains show the same growth pattern (data not shown).

All recombinant strains were analysed for phytase secretion using a phenotypic assay based on the degradation of insoluble Ca-phytate in solid medium (Fig 2). Clear zones of Ca-phytate degradation were observed for *B. bifidum* S17 strains harbouring pMgapS0P, pMgapS1P, pMgapS3P, pMgapS4P, and pMgapS6. By contrast, strains harbouring plasmids pMgapS2P and pMgapS5P did not display Ca-phytate degradation above background levels (pMgapP). A similar pattern of Ca-phytate degradation was observed for *B. longum* E18 strains, however at somewhat lower levels.

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Table 2. List of *B. bifidum* S17 proteins with predicted extracellular localization and information on the signal peptides identified in their amino acid sequences.

| Locus tag   | predicted function                                      | PsortB E-score | PsortB SP detection | SignalP D-score |
|-------------|---------------------------------------------------------|----------------|---------------------|-----------------|
| BBIF_0022   | Alpha-L-arabinofuranosidase                             | 9.26           | +                   | 0.718           |
| BBIF_0048   | 1,4-beta-N-acetylmuramidase                            | 9.98           | +                   | 0.732           |
| BBIF_0108   | hypothetical protein BBIF_0108                         | 8.91           | -                   | 0.157           |
| BBIF_0158   | Trypsin-like serine protease                            | 8.91           | -                   | 0.098           |
| BBIF_0246   | peptidylprolyl isomerase, FKBP-type                     | 9.26           | +                   | 0.556           |
| BBIF_0285   | hypothetical protein containing multiple sugar recognition domains | 9.76 | + | 0.886 |
| BBIF_0313   | hypothetical protein BBIF_0313                         | 8.91           | -                   | 0.191           |
| BBIF_0405   | hypothetical protein with CHAP domain                  | 8.91           | -                   | 0.408           |
| BBIF_0483   | conserved protein with the pectin lyase fold domain     | 9.13           | +                   | 0.723           |
| BBIF_0507   | beta-galactosidase BbgIII                              | 7.74           | +                   | 0.665           |
| BBIF_0538   | hypothetical protein BBIF_0538                         | 8.91           | -                   | 0.151           |
| BBIF_1193   | serine/cysteine peptidase                              | 9.13           | +                   | 0.836           |
| BBIF_1317   | alpha-L-fucosidase                                     | 9.97           | +                   | 0.714           |
| BBIF_1380   | hypothetical protein BBIF_1380                         | 9.13           | +                   | 0.831           |
| BBIF_1391   | D-alanyl-D-alanine carboxypeptidase                     | 9.72           | -                   | 0.432           |
| BBIF_1399   | hypothetical protein BBIF_1399                         | 8.91           | -                   | 0.129           |
| BBIF_1426   | hypothetical protein with NlpC/P60 domain              | 9.73           | +                   | 0.633           |
| BBIF_1427   | hypothetical protein containing CHAP domain            | 9.72           | -                   | 0.300           |
| BBIF_1457   | Rhs family protein                                     | 8.91           | -                   | 0.134           |
| BBIF_1458   | hypothetical protein BBIF_1458                         | 8.91           | -                   | 0.125           |
| BBIF_1461   | beta-N-acetylglucosaminidase                           | 9.76           | +                   | 0.760           |
| BBIF_1576   | beta-N-acetylglucosaminidase                           | 9.98           | +                   | 0.680           |
| BBIF_1733   | sialidase                                              | 9.97           | +                   | 0.792           |
| BBIF_1734   | sialidase                                              | **9.98**       | +                   | **0.862**       |
| BBIF_1740   | Alkaline phosphatase                                   | 9.73           | +                   | 0.776           |

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Secretion Reporter in Bifidobacteria

A

\[ P_{gap} \quad XhoI \]

\[ rep \quad appA \quad (pMB1) \]

\[ spc \quad repA \quad repB \]

HindIII

\[ pM\text{gapSOP} \]

5815 bps

B

![Graph A](image)

C

![Graph B](image)

RPU/ml

0.25

0.20

0.15

0.10

0.05

0.0

0

10

20

time [h]

S17 pM\text{gapSOP} • S17 pM\text{gapP}

RPU/mg

0.4

0.3

0.2

0.1

0

0

10

20

time [h]

S17 pM\text{gapSOP} • S17 pM\text{gapP}
In order to get a more quantitative comparison of protein secretion effected by the different SPs, phytase activity was measured in the supernatants of all strains generated. Using S0, highest levels of phytase activity in supernatants of \textit{B. bifidum} S17 were observed in stationary growth phase (Fig 1). The sequence upstream of the SP-\textit{appA} constructs including the promoter (P\textit{gap}) and all elements relevant for translation, i.e. ribosome binding site, start codon, and the sequence and distance between them, were identical in all strains. Thus, equal levels of expression at least amongst derivatives of the same strain was assumed and samples were collected from cultures grown for 16 h, i.e. early stationary growth phase, for determination of phytase activity. For \textit{B. bifidum} S17 strains, highest levels of phytase activity were observed for S6 and somewhat lower yet still efficient secretion was observed when S0, S1 and S4 were used as SP (Fig 3A). Statistical analysis suggests that protein secretion mediated by S4 and S6 is significant different (higher) compared to all other SPs and no difference is observed between S0 and S1 (Fig 3A; S3 Table). In \textit{B. longum} E18, S0 and S1 were the most efficient secretion signals (Fig 3B). Again no statistically significant difference between S0 and S1 was observed but both SPs differed from all other SPs (Fig 3B; S3 Table).

\textbf{Fig 1. Phytase reporter system to monitor protein secretion by bifidobacteria.} (A) Schematic representation of the secretion reporter plasmid pMgapS0P. The vector was constructed by fusing the SP of BBIF\_1734 (S0) to the phytase gene \textit{appA} of \textit{E. coli} K12 and cloning of the construct under the control of the gap promoter (P\textit{gap}) of \textit{B. bifidum} S17 in the vector backbone of pMDY23-P\textit{gap} using XhoI and HindIII. Relevant other features are: \textit{rep} (origin of replication for \textit{E. coli}), \textit{repAB} (origin of replication for bifidobacteria), \textit{spc} (spectinomycin resistance gene). (B)+(C) Phytase activity in supernatants (B) or crude extracts (C) of \textit{B. bifidum} S17/pMgapP (S17 pMgapP) and \textit{B. bifidum} S17/pMgapS0P (S17 pMgaS0P) during growth in RCM batch cultures. Values are relative phytase units (RPU) per ml supernatant (B) or mg protein in crude extracts (C) and are mean +/- standard deviation of three independent cultures measured in technical triplicates.

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\begin{table}[!h]
\centering
\footnotesize
\begin{tabular}{|l|l|l|l|l|}
\hline
Signal peptide & locus tag, predicted function & PsortB E-score \textsuperscript{a} & SignalP D-score \textsuperscript{b} & predicted cleavage site \textsuperscript{c} \\
\hline
\textbf{Sec-dependent signal peptides} & & & & \\
S0 & BBIF\_1734, sialidase & 9.98 & 0.862 & aa 33–37: ASA*AS \\
S3 & BLONG\_1728, hypothetical secreted protein with NlpC/P60 domain & 3.33 & 0.891 & aa 27–31: ATA*AE \\
S4 & BLONG\_0476, conserved hypothetical secreted protein with CHAP domain & 3.33 & 0.838 & aa 32–36: AQA*DT \\
S5 & BBIF\_1681, subtilisin family peptidase (lactocepin) & 9.98 & 0.798 & aa 26–30: ALA*AP \\
S6 & BBIF\_1761, surface protein with Gram positive anchor and Cna protein B-type domains & 0 \textsuperscript{d} & 0.879 & aa 29–33: ANA*AD \\
\hline
\textbf{Tat-dependent signal peptides} & & & & \\
S1 & BLONG\_0223, Tat-secreted glycosidase & 4.31 & 0.865 & aa 30–34: AQA*AD \\
S2 & BLONG\_1620, putative Tat-secreted pectin lyase-like protein & 9.13 & 0.772 & aa 28–32: AFA*QS \\
\hline
\end{tabular}
\caption{Signal peptides used to establish or tested in the phytase reporter assay and corresponding information on the predicted localization.}
\end{table}

\textsuperscript{a} E-score, extracellular score, calculated by cPSORTdb (version 3)

\textsuperscript{b} D-score, discrimination score, calculated by SignalP 4.1.

\textsuperscript{c} amino acid positions (aa), the sequence and the exact cleavage site (*) of the predicted SPs are indicated

\textsuperscript{d} This SP belong to a protein which is predicted to be located in the cell wall. The respective C-score for cell wall localization calculated by PSORTdb was 9.26.

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Secretion of cytosine deaminase

In order to demonstrate applicability of the identified SPs for secretion of a therapeutically relevant protein, we aimed at implementing a PCE approach using CD. For this purpose, we selected S0 based on the efficient secretion of phytase by both *B. bifidum* S17 and *B. longum* E18. The coding sequence of S0 was fused to the *codA* gene of *E. coli* K12. The fusion was cloned under control of P\_gap resulting in pAO-S0\_CD, which was transformed into *B. bifidum* S17. As a control, *codA* was cloned under control of P\_gap without a SP in the same vector backbone (pAO-CD). Growth of all strains was comparable in the absence of 5-FC (data not shown). Both recombinant strains as well as the wildtype were tested for growth inhibition by the prodrug 5-FC (Fig 4). This revealed that *B. bifidum* S17 showed good resistance to the prodrug and growth was only inhibited at the highest prodrug concentration tested (5 mg/ml). Expression of CD without a SP markedly increased sensitivity of the recombinant strain (*B. bifidum* S17/pAO-CD). Growth of this strain was already inhibited at 0.005 mg/ml 5-FC. By contrast, growth of *B. bifidum* S17/pAO-S0\_CD was comparable to that of the wild type in the presence of up to 0.1 mg/ml 5-FC. A slight inhibition of growth was observed only at 5-FC concentrations of 0.5 mg/ml and above but final OD\textsubscript{600} were higher than that of the strain.

| plasmid   | predicted pathway | B. bifidum S17 | B. longum E18 |
|-----------|-------------------|----------------|---------------|
| -         | -                 | ![Image](image1) | ![Image](image2) |
| pMgapP    | -                 | ![Image](image3) | ![Image](image4) |
| pMgapS0P  | Sec               | ![Image](image5) | ![Image](image6) |
| pMgapS1P  | Tat               | ![Image](image7) | ![Image](image8) |
| pMgapS2P  | Tat               | ![Image](image9) | ![Image](image10) |
| pMgapS3P  | Sec               | ![Image](image11) | ![Image](image12) |
| pMgapS4P  | Sec               | ![Image](image13) | ![Image](image14) |
| pMgapS5P  | Sec               | ![Image](image15) | ![Image](image16) |
| pMgapS6P  | Sec               | ![Image](image17) | ![Image](image18) |

Fig 2. Ca-phytate degradation of recombinant bifidobacteria expressing phytase with different signal peptides. Calcium phytate degradation by recombinant strains of *B. bifidum* S17 (A) and *B. longum* E18 (B) harbouring pMgapP-derived plasmids containing different SPs (S0-S6). The control plasmid pMgapP contains no SP and serves as a background control for expression of a non-secreted phytase. Overnight cultures of all strains were spotted in triplicate on RCM agar supplemented with 0.15% calcium phytate and imaged after anaerobic incubation for 48 h at 37°C. One representative spot of three independent cultures is shown.

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expressing the SP-less CD at all concentrations tested. Thus, the sensitivity to 5-FC was reduced by expressing CD as a secreted protein by two orders of magnitude suggesting efficient protein export.

**Discussion**

A number of traits of bacteria require extracellular proteins. These traits include acquisition of nutrients e.g. by secretion of glycosyl hydrolases for degradation of polysaccharides [42]. Extracellular proteins are not only required for mere survival, but are also involved in the interaction with the host. Thus, survival and interaction with the host of most bacteria crucially depends on secretion of functional proteins. Bifidobacteria as members of the normal human gut microbiota are no exception to this rule. In fact, in the highly competitive environment of the
gastrointestinal tract, secretion of proteins might be even more important than in other less densely populated habitats [42].

The vast majority of bacterial proteins are transported by the Sec pathway. Tat-dependent secretion is essential in only a few bacteria [25–27] and was shown to be important for virulence of a wide range of bacterial pathogens [43]. All Bifidobacterium sp. genomes analysed harboured genes for a Sec translocon. By contrast, only the B. longum genomes contained genes for Tat protein export machineries. This is consistent with the conservation and distribution of Sec and Tat systems amongst bacteria.

Various bifidobacterial signal sequences have been used for expression of secreted recombinant proteins. These include the SPs of the galacto-N-biose/lacto-N-biose I-binding protein [44] and exo-xylanase [45–47] of B. longum, the β-galactosidase of B. bifidum [48], and Sec2 and ApuB of B. breve [49,50]. However, in none of these cases the authors provide a rationale for selecting the respective SP and in most cases the sequences have not been analysed. Comparative and systematic studies on secretion systems and signal peptides of bifidobacteria are largely missing. In one study, a nuclease reporter was used to screen a genomic library of B. breve UCC2003 for signal sequences in E. coli and subsequent confirmation of positive clones in L. lactis [29]. This identified three Sec-dependent SPs and three signal sequences of putative membrane proteins. Further analysis of these SPs confirmed that the three Sec-dependent SPs are functional for secretion but quantitative analysis of nuclease activity in the supernatants showed no differences in efficiency of protein export.

We used a slightly different approach by selecting SPs of potentially secreted proteins of different bifidobacterial species predicted in silico. Using the predicted SPs of the sialidase BBIF_1734, we were able to establish a reporter system for protein secretion in bifidobacteria employing the phytase AppA of E. coli lacking its native SP. Heterologous expression and secretion of a phytase has previously been demonstrated in lactic acid bacteria [51,52].
relative levels of extra- and intracellular phytase activity in *B. bifidum* S17/pMgapS0P compared to *B. bifidum* S17/pMgapP (the strain expressing AppA without SP) suggest that S0 mediates efficient secretion of phytase. Although it is not possible to quantitatively compare levels of intracellular and extracellular levels of phytase due to intrinsic limitations of the Phytex method (RPU/mg of protein in crude extracts vs. RPU/ml supernatant), our results demonstrate that phytase is a valuable reporter system for the identification and analysis of secretion signals in bifidobacteria. The enzyme is resistant against proteases, active in a wide range of pH values, and activity is optimal at pH 4–5, which is the normal pH in stationary phase batch fermentations of bifidobacteria [53].

Bifidobacteria do not encode *appA* homologues since BLAST searches revealed no significant hits in the genus *Bifidobacterium* (data not shown). Nevertheless, phytase activity has been detected in different *Bifidobacterium* sp. [54,55]. Recently, two enzymes of bifidobacteria with phytase activity have been characterized. Based on sequence comparisons, the enzymes belong to a different phylogenetic cluster than the *E. coli* AppA enzyme and are more closely related to the phytases of plants, fungi and vertebrates [56]. Intrinsic activity of non-AppA phytases or other phosphatases might explain the slight background observed for *B. bifidum* S17/pMgapP.

Using the phytase reporter, we were able to screen a number of other SPs of potentially secreted proteins of *B. bifidum* S17 and *B. longum* E18. All strains were initially screened for secreted phytase by detection of Ca-phytate degradation in agar plates and measuring phytase activity in culture supernatants. Some of the SPs did not produce zones of phytate degradation on agar, but phytase activity well above background was measured in the supernatants. Thus, Ca-phytate degradation in agar plates is rather a first, but not definite, indicator for the functionality of highly efficient SPs and measuring phytase activity in supernatants allows a more quantitative analysis.

All SPs analysed yielded phytase activities above background in both *B. bifidum* S17 and *B. longum* E18, suggesting that bifidobacterial SPs might be functional in other *Bifidobacterium* sp. besides their original hosts. Despite their annotation as (putative) Tat substrates, SPs S1 and S2 mediated phytase export in *B. bifidum* S17, which lacks a Tat system. Further *in silico* analysis revealed that both SPs yielded no prediction for Tat recognition using TATFIND and no Tat motifs were found by TatP (data not shown). Moreover, S1 and S2 had D-scores of 0.865 and 0.772, respectively, in the SignalP analysis. Collectively, this suggests that the predicted Tat-dependence of these SPs might be false and they might actually be Sec-dependent secretion signals.

Amongst the tested SPs, the SP of BBIF_1734 (S0) mediated efficient protein secretion in both *B. bifidum* S17 and *B. longum* E18. Thus, we selected S0 to clone a vector for expression and secretion of a therapeutically relevant protein. A number of bacteria including bifidobacteria are investigated as gene delivery vectors for cancer therapy. One of the approaches pursued, is the so-called Bacterial Directed Enzyme Prodrug Therapy (BDEPT), i.e. the use of recombinant bacteria expressing secreted enzymes for conversion of non-toxic prodrugs into their active, cytotoxic form [24]. An enzyme frequently used in BDEPT is the cytosine deaminase [24]. This enzyme is present in a wide range of microorganisms but not in mammalian cells. Its physiological role is deamination of cytosine to uracil and, as a non-specific, non-physiologic side reaction, also catalyses conversion of the prodrug 5-FC to the tumour therapeutic 5-FU.

In a proof-of-principle approach, a construct was generated for expression of a secreted form of the CodA cytosine deaminase of *E. coli* in bifidobacteria using the P*gap* promoter and S0 to achieve secretion. *B. bifidum* S17/pAO-S0P tolerate doses of 5-FC of 0.2 mg/ml without inhibition of growth. By contrast, growth of the isogenic strain expressing a non-secreted CD is
inhibited at concentration of 0.005 mg/ml and above. This demonstrates that a therapeutically relevant protein can be expressed and secreted in its active form using the SP of BBIF_1734. Moreover, the highest systemic concentration of 5-FC that is tolerated during therapy without adverse side effects is 0.1 mg/ml [57]. Thus, expression of CD as a secreted form by B. bifidum S17 renders this strain tolerant to therapeutically relevant doses.

A number of genetically engineered bifidobacteria expressing various therapeutic genes were successfully used to inhibit tumour growth in mouse models [58–62]. CD has also been expressed in bifidobacteria [63,64] and one of these recombinant strains was successfully used in combination with 5-FC to inhibit growth of a subcutaneous tumours of a melanoma cell line in mice [64]. Expression CD and other proteins in secreted form by bifidobacteria using appropriate secretion signals might improve their therapeutic efficacy.

In summary, our data shows that we have successfully developed a reporter system for identification and analysis of secretion signals in bifidobacteria. We furthermore demonstrated that an SP identified using this report is functional in mediating secretion of a therapeutically relevant protein. Rationally designed systems for secreted proteins might improve the efficacy of recombinant bifidobacteria as probiotic supplements in functional foods or in therapeutic applications such as cancer therapy.

Supporting Information

S1 Data. Amino acid sequences of Sec and Tat homologues of representative Bifidobacterium sp. (DOCX)

S1 Table. Bacterial strains. (DOCX)

S2 Table. Oligonucleotides. (DOCX)

S3 Table. Statistical analysis of phytase secretion using different signal peptides. (DOCX)

Author Contributions

Conceived and designed the experiments: ZS CUR. Performed the experiments: AO CW ZS. Analyzed the data: AO CW ZS CUR. Wrote the paper: AO ZS CUR.

References

1. Kleerebezem M, Vaughan EE (2009) Probiotic and gut lactobacilli and bifidobacteria: molecular approaches to study diversity and activity. Annu Rev Microbiol 63: 269–290. Available: http://www.ncbi.nlm.nih.gov/pubmed/19575569. doi: 10.1146/annurev.micro.091208.073341 PMID: 19575569

2. Gareau MG, Sherman PM, Walker WA (2010) Probiotics and the gut microbiota in intestinal health and disease. Nat Rev Gastroenterol Hepatol 7: 503–514. Available: http://www.ncbi.nlm.nih.gov/pubmed/20664519. doi: 10.1038/nrgastro.2010.117 PMID: 20664519

3. Schell MA, Karmirantzou M, Snel B, Vilanova D, Berger B, Pessi G, et al. (2002) The genome sequence of Bifidobacterium longum reflects its adaptation to the human gastrointestinal tract. Proc Natl Acad Sci U S A 99: 14422–14427. Available: http://www.ncbi.nlm.nih.gov/pubmed/12381787. PMID: 12381787

4. Ivanov D, Emonet C, Foata F, Affolter M, Delley M, Fisseha M, et al. (2006) A serpin from the gut bacterium Bifidobacterium longum inhibits eukaryotic elastase-like serine proteases. J Biol Chem 281: 17246–17252. Available: http://www.ncbi.nlm.nih.gov/pubmed/16627467. PMID: 16627467

5. Turroni F, Foroni E, O’Conneill Motherway M, Bottacini F, Giubellini V, Zomer A, et al. (2010) Characterization of the serpin-encoding gene of Bifidobacterium breve 210B. Appl Environ Microbiol 76: 3206–3219. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2889134&tool =
Marcobal A, Sonnenburg JL (2012) Human milk oligosaccharide consumption by intestinal microbiota.

14. González-Rodríguez I, Ruiz L, Gueimonde M, Margolles A, Sánchez B (2013) Factors involved in the

15. Yazawa K, Fujimori M, Amano J, Kano Y, Taniguchi S (2000) Bifidobacterium longum as a delivery sys-

16. Grimm V, Westermann C, Riedel CU (2014) Bifidobacteria-Host Interactions-An Update on Colonisa-

17. Kimura NT, Taniguchi S, Aoki K, Baba T (1980) Selective localization and growth of Bifidobacterium

18. Cronin M, Akin AR, Collins SA, Meganck J, Kim J-H, Baban CK, et al. (2012) High resolution in vivo bio-

19. Cronin M, Akin AR, Collins SA, Meganck J, Kim J-H, Baban CK, et al. (2010) Oral administration of bifidobac-

20. Cronin M, Akin AR, Collins SA, Meganck J, Kim J-H, Baban CK, et al. (2012) Oral delivery of bifidobacteria

pmcentre&rendertype = abstract. Accessed 24 October 2014. doi: 10.1128/AEM.02938-09 PMID:

20348296

6. Alvarez-Martin P, O’Connell Motherway M, Turroni F, Foroni E, Ventura M, van Sinderen D (2012) A
two-component regulatory system controls autoregulated serpin expression in Bifidobacterium breve

UCC2003. Appl Environ Microbiol 78: 7032–7041. Available: http://www.ncbi.nlm.nih.gov/pubmed

22843530. Accessed 10 June 2014. doi: 10.1128/AEM.01776-12 PMID: 22843530

7. Ewaschuk JB, Diaz H, Meddings L, Diederichs B, Dmytrash A, Backer J, et al. (2008) Secreted bioac-
tive factors from Bifidobacterium infantis enhance epithelial cell barrier function. Am J Physiol Gastroint-
est Liver Physiol 295: G1025–G1034. Available: http://www.ncbi.nlm.nih.gov/pubmed/18787064. doi:

10.1152/ajpgi.90227.2008 PMID: 18787064

8. Turroni F, Bottacini F, Foroni E, Mulder I, Kim J-H, Zomer A, et al. (2010) Genome analysis of Bifidobac-
terium bifidum PRL2010 reveals metabolic pathways for host-derived glycans foraging. Proc Natl Acad

Sci U S A 107: 19514–19519. Available: http://www.ncbi.nlm.nih.gov/pubmed/20974960. doi: 10.

1073/pnas.101100107 PMID: 20974960

9. LoCascio RG, Desai P, Sela DA, Weimer B, Mills DA (2010) Broad conservation of milk utilization

genes in Bifidobacterium longum subsp. infantis as revealed by comparative genomic hybridization.

Appl Environ Microbiol 76: 7373–7381. Available: http://www.pubmedcentral.nih.gov/articlerender.
fg?artid=2976205&tool = pmcentre&rendertype = abstract. Accessed 21 March 2014, doi: 10.1128/ 

AEM.00675-10 PMID: 20802066

10. Sela DA, Garrido D, Lerno L, Wu S, Tan K, Eom HJ, et al. (2012) Bifidobacterium longum subsp. infantis

ATCC 15697 α-fucosidases are active on fucosylated human milk oligosaccharides. Appl Environ

Microbiol 78: 795–803. Available: http://www.ncbi.nlm.nih.gov/pubmed/22138995. Accessed 21

March 2014, doi: 10.1128/AEM.06762-11 PMID: 22138995

11. O’Connell Motherway M, Fitzgerald GF, Neirynck S, Ryan S, Steidler L, van Sinderen D (2008) Charac-
terization of ApuB, an extracellular type II amylopullulanase from Bifidobacterium breve UCC2003.

Appl Environ Microbiol 74: 6271–6279. Available: http://www.ncbi.nlm.nih.gov/pubmed/18689518, doi:

10.1128/AEM.01169-08 PMID: 18689518

12. Bottacini F, Ventura M, van Sinderen D, O’Connell Motherway M (2014) Diversity, ecology and intesti-
nal function of bifidobacteria. Microb Cell Fact 13 Suppl 1: S4. Available: http://www.pubmedcentral.

nih.gov/articlerender.fcgi?artid=4155821&tool = pmcentre&rendertype = abstract. Accessed 14 April

2015, doi: 10.1186/1475-2859-13-S1-S4 PMID: 2518714

13. Sela DA, Mills DA (2010) Nursing our microbiota: molecular linkages between bifidobacteria and milk

oligosaccharides. Trends Microbiol 18: 298–307. Available: http://www.pubmedcentral.nih.gov/

articlerender.fcgi?artid=2902656&tool = pmcentre&rendertype = abstract. Accessed 14 April 2010.

doi: 10.1164/tim.2010.03.008 PMID: 20409714

14. Marcobal A, Sonnenburg JL (2012) Human milk oligosaccharide consumption by intestinal microbiota.

Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis 18 Suppl 4: 12–15. Available: http://

www.ncbi.nlm.nih.gov/pubmed/22647041.

15. González-Rodríguez I, Ruiz L, Gueimonde M, Margolles A, Sánchez B (2013) Factors involved in the

colonization and survival of bifidobacteria in the gastrointestinal tract. FEMS Microbiol Lett 340: 1

265–274. Available:http://www.ncbi.nlm.nih.gov/pubmed/10770636. PMID:10770636

16. Grimm V, Westermann C, Riedel CU (2014) Bifidobacteria-Host Interactions-An Update on Coloniisa-
tion Factors. Biomed Res Int 2014: 960826. Available: http://www.pubmedcentral.nih.gov/articlerender.
fg?artid=417770&tool = pmcentre&rendertype = abstract. Accessed 20 October 2014. doi: 10.1155/2014/960826 PMID: 25295282

17. Kimura NT, Taniguchi S, Aoki K, Baba T (1980) Selective localization and growth of Bifidobacterium

bifidum in mouse tumors following intravenous administration. Cancer Res 40: 2061–2068. Available:

http://www.ncbi.nlm.nih.gov/pubmed/6989495. PMID: 6989495

18. Yazawa K, Fujimori M, Amano J, Kano Y, Taniguchi S (2000) Bifidobacterium longum as a delivery system
during cancer gene therapy: selective localization and growth in hypoxic tumors. Cancer Gene Ther 7: 

269–274. Available: http://www.ncbi.nlm.nih.gov/pubmed/10770636. PMID: 10770636

19. Cronin M, Morrissey D, Rajendran S, El Mashad SM, van Sinderen D, O’Sullivan GC, et al. (2010) 

Orally administered bifidobacteria as vehicles for delivery of agents to systemic tumors. Mol Ther 18: 

1397–1407. Available: http://www.ncbi.nlm.nih.gov/pubmed/20389288. Accessed 30 January 2014. 
doi: 10.1038/mt.2010.59 PMID: 20389288

20. Cronin M, Akin AR, Collins SA, Meganck J, Kim J-H, Baban CK, et al. (2012) High resolution in vivo bio-
luminescent imaging for the study of bacterial tumour targeting. PLoS One 7: e308940. Available: http://

www.ncbi.nlm.nih.gov/pubmed/22295120. Accessed 30 January 2014. doi: 10.1371/journal.pone. 
0030940 PMID: 22295120
21. Forbes NS (2010) Engineering the perfect (bacterial) cancer therapy. Nat Rev Cancer 10: 785–794. Available: http://www.ncbi.nlm.nih.gov/pubmed/20944664. doi: 10.1038/nrc2934 PMID: 20944664

22. Cronin M, Stanton RM, Francis KP, Tangney M (2012) Bacterial vectors for imaging and cancer gene therapy: a review. Cancer Gene Ther 19: 731–740. Available: http://www.ncbi.nlm.nih.gov/pubmed/22996740. Accessed 30 January 2014. doi: 10.1038/cgt.2012.59 PMID: 22996740

23. Baban CK, Cronin M, O’Hanlon D, O’Sullivan GC, Tangney M (2010) Bacteria as vectors for gene therapy of cancer. Bioeng Bugs 1: 385–394. Available: http://www.ncbi.nlm.nih.gov/pubmed/21468205. doi: 10.4161/bbug.1.6.13146 PMID: 21468205

24. Lehouritis P, Springer C, Tangney M (2013) Bacterial-directed enzyme prodrug therapy. J Control Release 170: 120–131. Available: http://www.ncbi.nlm.nih.gov/pubmed/23688772. Accessed 29 January 2015. doi: 10.1016/j.jconrel.2013.05.005 PMID: 23688772

25. Du Plessis DJF, Nouwen N, Driessen AJM (2011) The Sec translocon. Biochim Biophys Acta 1808: 851–865. Available: http://www.ncbi.nlm.nih.gov/pubmed/20801097. Accessed 15 December 2014. doi: 10.1016/j.bbapap.2010.08.016 PMID: 20801097

26. Palmer T, Berks BC (2012) The twin-arginine translocation (Tat) protein export pathway. Nat Rev Microbiol 10: 483–496. Available: http://www.ncbi.nlm.nih.gov/pubmed/22683878. Accessed 21 November 2014. doi: 10.1038/nrmicro2814 PMID: 22683878

27. Natale P, Brüser T, Driessen AJM (2008) Sec- and Tat-mediated protein secretion across the bacterial cytoplasmic membrane—distinct translocases and mechanisms. Biochim Biophys Acta 1778: 1735–1756. Available: http://www.ncbi.nlm.nih.gov/pubmed/17935691. Accessed 20 January 2015. PMID: 17935691

28. Goosens VJ, Monteferrante CG, van Dijl JM (2014) The Tat system of Gram-positive bacteria. Biochim Biophys Acta 1843: 1698–1706. Available: http://www.ncbi.nlm.nih.gov/pubmed/24140208. Accessed 2 February 2015. doi: 10.1016/j.bbamcr.2013.10.008 PMID: 24140208

29. MacConaill LE, Fitzgerald GF, Van Sinderen D (2003) Investigation of protein export in Bifidobacterium breve UCC2003. Appl Environ Microbiol 69: 6994–7001. Available: http://www.ncbi.nlm.nih.gov/pubmed/14660341. PMID: 14660341

30. Grimm V, Gleinser M, Neu C, Zhurina D, Riedel CU (2014) Expression of fluorescent proteins in bifidobacteria expressing the Bifidobacterium bifidum-specific lipoprotein BopA. Microb Cell Fact 13: 246. Available: http://www.ncbi.nlm.nih.gov/pubmed/24584243. PMID: 24584243

31. Heckman KL, Pease LR (2007) Gene splicing and mutagenesis by PCR-driven overlap extension. Nat Protoc 2: 924–932. Available: http://www.ncbi.nlm.nih.gov/pubmed/17446874. Accessed 9 July 2014. PMID: 17446874

32. Gleinser M, Grimm V, Zhurina D, Yuan J, Riedel CU (2012) Improved adhesive properties of recombinant bifidobacteria expressing the Bifidobacterium bifidum-specific lipoprotein BopA. Microb Cell Fact 11: 80. Available: http://www.ncbi.nlm.nih.gov/pubmed/22694891. doi: 10.1186/1475-2859-11-80 PMID: 22694891

33. Sun Z, He X, Brancaccio VF, Yuan J, Riedel CU (2014) Bifidobacteria Exhibit LuxS-Dependent Autoinducer 2 Activity and Biofilm Formation. PLoS One 9: e88260. Available:http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3013690&tool = pmcentrez&rendertype = abstract. Accessed 10 February 2015. doi: 10.1371/journal.pone.0088260 PMID: 24505453

34. Kim TW, Lei XG (2005) An improved method for a rapid determination of phytase activity in animal feed. J Anim Sci 83: 1062–1067. Available: http://www.ncbi.nlm.nih.gov/pubmed/15827251. Accessed 5 January 2015. PMID: 15827251

35. Lambrechts C, Boze H, Moulin G, Galzy P (1992) Utilization of phytate by some yeasts. Biotechnol Lett 14: 61–66. Available: http://dx.doi.org/10.1007/BF01030915.

36. Yu NY, Laird MR, Spencer C, Brinkman FSL (2011) PSORTdb—an expanded, auto-updated, user-friendly protein subcellular localization database for Bacteria and Archaea. Nucleic Acids Res 39: D241–D244. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3013690&tool = pmcentrez&rendertype = abstract. Accessed 4 January 2015. doi: 10.1093/nar/gkq1093 PMID: 21071402

37. Petersen TN, Brunak S, von Heijne G, Nielsen H (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods 8: 785–786. Available: http://www.ncbi.nlm.nih.gov/pubmed/21595131. doi: 10.1038/nmeth.1701 PMID: 21595131

38. Bendtsen JD, Nielsen H, Widdick D, Palmer T, Brunak S (2005) Prediction of twin-arginine signal peptides. BMC Bioinformatics 6: 167. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1182353&tool = pmcentrez&rendertype = abstract. Accessed 9 October 2014. PMID: 15992409

39. Rose RW, Brüser T, Kissinger JC, Pohlschröder M (2002) Adaptation of protein secretion to extremely high-salt conditions by extensive use of the twin-arginine translocation pathway. Mol Microbiol 45:
40. Shkoporov AN, Efimov BA, Khokhlova E V, Kafarskaia LI, Smeianov V V (2008) Production of human Aisaka K, Igarashi A, Uwajima T (1991) Purification, crystallization, and characterization of neuraminidase from Micromonospora viridifaciens. Agric Biol Chem 55: 997–1004. Available: https://www.jstage.jst.go.jp/article/bbb1961/55/4/55_4_997/_article. Accessed 5 December 2014.

41. White BA, Lamed R, Bayer EA, Flint HJ (2014) Biomass utilization by gut microbiomes. Annu Rev Microbiol 68: 279–296. Available: http://www.ncbi.nlm.nih.gov/pubmed/25002092. Accessed 26 January 2015. doi: 10.1146/annurev-micro-092412-155618 PMID: 25002092

42. De Buck E, Lammertyn E, Anné J (2008) The importance of the twin-arginine translocation pathway for bacterial virulence. Trends Microbiol 16: 442–453. Available: http://www.ncbi.nlm.nih.gov/pubmed/18175874. Accessed 8 January 2015. doi: 10.1016/j.tim.2008.06.004 PMID: 18175874

43. Yamamoto S, Wada J, Katayama T, Jikimoto T, Nakamura M, Kinoshita S, et al. (2010) Genetically modified Bifidobacterium displaying Salmonella-antigen protects mice from lethal challenge of Salmonella Typhimurium in a murine typhoid fever model. Vaccine 28: 6684–6691. Available: http://www.ncbi.nlm.nih.gov/pubmed/20065960. doi: 10.1038/j.ijo.2009.277 PMID: 20065960

44. Yao J, Wang J-Y, Lai M-G, Li Y-X, Zhu H-M, Shi R-Y, et al. (2011) Treatment of mice with dextran sulfate sodium-induced colitis with human interleukin 10 secreted by transformed Bifidobacterium longum. Mol Pharm 8: 488–497. Available: http://www.ncbi.nlm.nih.gov/pubmed/21271712. doi: 10.1021/mp100331r PMID: 21271712

45. Reyes Escogido ML, De León Rodríguez A, Barba de la Rosa AP (2007) A novel binary expression vector for production of human IL-10 in Escherichia coli and Bifidobacterium longum. Biotechnol Lett 29: 1249–1253. Available: http://www.ncbi.nlm.nih.gov/pubmed/17487549. PMID: 17487549

46. Shkoporov AN, Efimov BA, Khokhlova E V, Katarskaia LI, Smeianov V V (2008) Production of human basic fibroblast growth factor (FGF-2) in Bifidobacterium breve using a series of novel expression/secretion vectors. Biotechnol Lett 30: 1983–1989. Available: http://www.ncbi.nlm.nih.gov/pubmed/18575808. doi: 10.1007/s10529-008-9772-8 PMID: 18575808

47. Khokhlova E V, Efimov BA, Katarskaia LI, Shkoporov AN (2010) Heterologous expression of secreted biologically active human interleukin-10 in Bifidobacterium breve. Arch Microbiol 192: 769–774. Available: http://www.ncbi.nlm.nih.gov/pubmed/20631991. doi: 10.1007/s00203-010-0606-4 PMID: 20631991

48. Kerovuo J, Tynkkynen S (2000) Expression of Bacillus subtilis phytase in Lactobacillus plantarum 755. Lett Appl Microbiol 30: 325–329. Available: http://www.ncbi.nlm.nih.gov/pubmed/10792656. Accessed 29 January 2015. PMID: 10792656

49. Wang Lang, Yang Y, Cai B, Cao P, Yang M, Chen Y (2014) Coexpression and secretion of endoglucanase and phytase genes in Lactobacillus reuteri. Int J Mol Sci 15: 12842–12860. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4139877&tool = pmcentrez&rendertype = abstract. Accessed 29 January 2015. doi: 10.3390/ijms15071249 PMID: 250050780

50. Lei XG, Weaver JD, Mullaney E, Ullah AH, Azain MJ (2013) Phytase, a new life for an old enzyme. Annu Rev Anim Biosci 1: 283–309. Available: http://www.ncbi.nlm.nih.gov/pubmed/25387021. Accessed 25 January 2015. doi: 10.1146/annurev-animal-031412-103717 PMID: 25387021

51. Haros M, Bielecka M, Honke J, Sanz Y (2007) Myo-inositol hexakisphosphate degradation by Bifidobacterium infantis ATCC 15697. Int J Food Microbiol 117: 76–84. Available: http://www.ncbi.nlm.nih.gov/pubmed/17462768. Accessed 5 April 2015. PMID: 17462768

52. Palacios MC, Haros M, Rosell CM, Sanz Y (2008) Selection of phytate-degrading human bifidobacteria and application in whole wheat dough fermentation. Food Microbiol 25: 169–176. Available: http://www.ncbi.nlm.nih.gov/pubmed/17993391. Accessed 5 April 2015. PMID: 17993391

53. Tamayo-Ramos JA, Sanz-Penella JM, Yebra MJ, Monedero V, Haros M (2012) Novel phytases from Bifidobacterium pseudocatenulatum ATCC 27919 and Bifidobacterium longum subsp. infantis ATCC 15697. Appl Environ Microbiol 78: 5013–5015. Available: http://www.pubmedcentral.nih.gov/
57. Vermes A, Guchelaar HJ, Dankert J (2000) Flucytosine: a review of its pharmacology, clinical indications, pharmacokinetics, toxicity and drug interactions. J Antimicrob Chemother 46: 171–179. Available: http://www.ncbi.nlm.nih.gov/pubmed/10933638. Accessed 3 February 2015. PMID: 10933638

58. Zhu L-P, Yin Y, Xing J, Li C, Kou L, Hu B, et al. (2009) Therapeutic efficacy of Bifidobacterium longum-mediated human granulocyte colony-stimulating factor and/or endostatin combined with cyclophosphamide in mouse-transplanted tumors. Cancer Sci 100: 1986–1990. Available: http://www.ncbi.nlm.nih.gov/pubmed/19678823. doi: 10.1111/j.1349-7006.2009.01275.x PMID: 19678823

59. Zhu H, Li Z, Mao S, Ma B, Zhou S, Deng L, et al. (2011) Antitumor effect of sFlt-1 gene therapy system mediated by Bifidobacterium Infantis on Lewis lung cancer in mice. Cancer Gene Ther 18: 884–896. Available: http://www.ncbi.nlm.nih.gov/pubmed/21921942. doi: 10.1038/cgt.2011.57 PMID: 21921942

60. Hu B, Kou L, Li C, Zhu L-P, Fan Y-R, Wu Z-W et al. (2009) Bifidobacterium longum as a delivery system of TRAIL and endostatin cooperates with chemotherapeutic drugs to inhibit hypoxic tumor growth. Cancer Gene Ther 16: 655–663. Available: http://www.ncbi.nlm.nih.gov/pubmed/19229287. doi: 10.1038/cgt.2009.7 PMID: 19229287

61. Li X, Fu G-F, Fan Y-R, Liu W-H, Liu X-J, Wang J-J, et al. (2003) Bifidobacterium adolescentis as a delivery system of endostatin for cancer gene therapy: selective inhibitor of angiogenesis and hypoxic tumor growth. Cancer Gene Ther 10: 105–111. Available: http://www.ncbi.nlm.nih.gov/pubmed/12536198. PMID: 12536198

62. Xu Y-F, Zhu L-P, Hu B, Fu G-F, Zhang H-Y, Wang J-J, et al. (2007) A new expression plasmid in Bifidobacterium longum as a delivery system of endostatin for cancer gene therapy. Cancer Gene Ther 14: 151–157. Available: http://www.ncbi.nlm.nih.gov/pubmed/17068487. PMID: 17068487

63. Nakamura T, Sasaki T, Fujimori M, Yazawa K, Kano Y, Amano J, et al. (2002) Cloned cytosine deaminase gene expression of Bifidobacterium longum and application to enzyme/pro-drug therapy of hypoxic solid tumors. Biosci Biotechnol Biochem 66: 2362–2366. Available: http://www.ncbi.nlm.nih.gov/pubmed/12506973. PMID: 12506973

64. Yi C, Huang Y, Guo Z, Wang S (2005) Antitumor effect of cytosine deaminase/5-fluorocytosine suicide gene therapy system mediated by Bifidobacterium infantis on melanoma. Acta Pharmacol Sin 26: 629–634. Available: http://www.ncbi.nlm.nih.gov/pubmed/15842785. PMID: 15842785

65. Zhurina D, Dudnik A, Waidmann MS, Grimm V, Westermann C, Breitinger KJ, et al. (2013) High-Quality Draft Genome Sequence of Bifidobacterium longum E18, Isolated from a Healthy Adult. Genome Announc 1: pii: e01084–13. Available: http://www.ncbi.nlm.nih.gov/pubmed/24356845. Accessed 8 April 2014.

66. Zhurina D, Zomer A, Gleinser M, Brancaccio VF, AUCHTER M, Waidmann MS, et al. (2011) Complete genome sequence of Bifidobacterium bifidum S17. J Bacteriol 193: 301–302. Available: http://www.ncbi.nlm.nih.gov/pubmed/21037011. doi: 10.1128/jb.01180-10 PMID: 21037011

67. Bottacini F, O Connell Motherway M, Kuczynski J, O Connell KJ, Serafini F, Duranti S, et al. (2014) Comparative genomics of the Bifidobacterium breve taxon. BMC Genomics 15: 170. Available: http://www.ncbi.nlm.nih.gov/pubmed/24581150. Accessed 5 March 2014. doi: 10.1186/1471-2164-15-170 PMID: 24581150

68. Loquasto JR, Barrangou R, Dudley EG, Stahl B, Chen C, Roberts RF (2013) Bifidobacterium animalis subsp. lactis ATCC 27673 is a genomically unique strain within this conserved subspecies. Appl Environ Microbiol. Available: http://www.ncbi.nlm.nih.gov/pubmed/23995933.

69. Ventura M, Turroni F, Zomer A, Foroni E, Giubellini V, Bottacini F, et al. (2009) The Bifidobacterium dentium Bd1 genome sequence reflects its genetic adaptation to the human oral cavity. PLoS Genet 5: e1000785. Available: http://www.ncbi.nlm.nih.gov/pubmed/20041198. doi: 10.1371/journal.pgen.1000785 PMID: 20041198