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Identification of *Brevibacterium flavum* genes related to receptors involved in bacteriophage BFK20 adsorption

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ABSTRACT

Phage infection of bacterial cells is a process requiring the interaction between phage receptor binding proteins and receptors on the bacterial cell surface. We prepared a *Brevibacterium flavum* CCM 251 EZ-Tn5 transposon insertional library and isolated phage-resistant mutants. Analysis of the DNA fragments produced by single-primer PCR was used to determine the EZ-Tn5 transposon insertion sites in the genomes of phage-resistant *B. flavum* mutants. Seven disrupted genes were identified in forty *B. flavum* mutants. The phage resistance of these mutants was demonstrated by cultivation analysis in the presence of BFK20, and the adsorption rate of BFK20 to these mutants was tested. *B. flavum* mutants displayed significantly reduced adsorption rates; the lowest rate was observed for mutants containing interrupted major facilitator superfamily (MFS) protein and glycosyltransferase genes. Uninterrupted forms of these genes were cloned into corynebacterial vector pJUP06 and used for *in trans* complementation of the corresponding *B. flavum* mutants. The growth of these complemented mutants when infected with BFK20 closely resembled that of wild-type *B. flavum*. These complemented mutants also exhibited similar BFK20 adsorption as the wild-type control. We infer that the disrupted MFS protein and glycosyltransferase genes are responsible for the phage-resistant phenotype of these *B. flavum* transposition mutants.

1. Introduction

Bacteriophages (phages) are viruses specifically infecting bacteria. Phage infection of the host bacterial cell is a multistage process (Molineux, 2006). Infection begins with phages attaching to one or more receptors on the surface of their bacterial host using receptor binding proteins (RBPs), which are usually located on the distal end of the phage tail (Dunne et al., 2018; Dowah and Clokie, 2018). Through a series of interactions between the bacteriophage RBPs and the bacterial cell surface receptors, the virus recognizes a potentially sensitive host and positions itself for DNA injection. Each phage has its own particular host range. Phages can be limited to a narrow host range involving a particular microbial species or even strain (Rakhuba et al., 2010; Bertozzi Silva et al., 2016). Adsorption of bacteriophages to the cell surface generally consists of two steps: initial contact with reversible binding, followed by irreversible attachment. The first step involves random collisions between phage and host and subsequent reversible binding of the virion to bacterial surface components. In this stage adsorption is not definitive and the phage can desorb from the host. This process probably serves to keep the phage close to the cell surface as it searches for a specific receptor. The reversible interactions can be formed using one or two tail fibers as in *E. coli* phages T7 (*Podoviridae*) and T4 (*Myoviridae*) (Storms and Sauvageau, 2015). *Siphoviridae* such as λ., the *Bacillus subtilis* phage SPP1and the lactococcal phage p2 possess at the C-terminus of major tail protein an adhesin fold which appears as decorations on the surface of the tail. It has been suggested that such domains help the primary adhesion of phages to their host and probably play a similar role in adsorption as tail fibers of *Podoviridae* and *Myoviridae* (Spinelli et al., 2014). The transition to irreversible adsorption during the second step usually requires additional binding of the phage to either the same receptor found in the first step or to a second receptor. The specific connection between the bacterial receptor and phage binding domains is sometimes mediated by enzymatic cleavage followed by conformational rearrangements of the virion structure. These steps are necessary for creating a channel for phage genome delivery through the cell envelope (Bertozzi Silva et al., 2016; Letarov and Kulikov, 2017).

Many diverse molecular structures on the surface of bacteria can act as phage receptors, but their variety and position on the cell differs with particular bacteria–phage interactions. Several recent reviews provide an overview of the phage receptors identified so far (Bertozzi Silva et al., 2016; Letarov and Kulikov, 2017). The nature of the receptors is mostly determined by the composition of the host cell wall and surface layer structures. Phage receptors have been identified in the cell wall

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components of both Gram-positive and Gram-negative bacteria, in bacterial capsules or slime layers, and in pili and flagella (Rakhuba et al., 2010; Bertozzi Silva et al., 2016). Interactions in the phage-host system have been widely studied for phages that target Gram-negative bacteria and less is known about phages that infect Gram-positive bacteria (Bertozzi Silva et al., 2016). In Gram-negative bacteria, common phage receptors are lipopolysaccharides (LPS) and outer membrane proteins (Sørensen et al., 2011). The adsorption mechanisms have been thoroughly studied in the model *E. coli* viruses T4 (Goldberg et al., 1994) and T7 (Hu et al., 2013). The T4 phage attaches reversibly to LPS or to the outer membrane porin protein OmpC, depending on the *E. coli* strain. This attachment then leads to the irreversible binding of T4 with the LPS outer core region. Similarly, *E. coli* phage T7 also irreversibly binds to LPS (Sørensen et al., 2011; González-García et al., 2015). Some other phages also use LPS as both primary and secondary receptors, e.g. the *Salmonella enterica* P22 phage (Letarov and Kulikov, 2017).

For phages that infect Gram-positive bacteria, peptidoglycan (PG) and cell wall polysaccharides (Bertozzi Silva et al., 2016) are common receptors. Many phages bind to PG together with other cell wall components, most often wall teichoic acids (WTA) or lipoteichoic acids (LTA). Binding to PG together with WTA recognition were shown for the *Listeria* phage A511 (Habann et al., 2014) and the *Staphylococcus aureus* phage phi11 (Li et al., 2016a). Many phages use only teichoic acids as receptors, *B. subtilis* phages use the D-glucose chain of cell surface teichoic acids (Rakhuba et al., 2010), other use lipoteichoic acid (Räisänen et al., 2004; Mahony et al., 2017**)**. A repository of current information about identified host receptors is PhReD (www.ualberta.ca/phred), an open-access, updatable database (Bertozzi Silva et al., 2016).

Bertozzi Silva et al. (2016) have described how phages have exploited the receptors presently known in the three Caudovirales families. The *Siphoviridae* of Gram-negative bacteria require proteinaceous receptors for adsorption while all but one identified *Siphoviridae* of Gram-positive bacteria require saccharides. *Myoviridae* of both Gram-negative and Gram-positive bacteria mostly bind to sugar moieties. Finally, all *Podoviridae* surveyed so far require polysaccharides for adsorption.

Understanding how phages that target Gram-positive bacteria recognize their host and adsorb to the cell surface using specific cell wall receptors is crucial for understanding phage host range, for developing novel detection and biocontrol tools, and for evaluating the efficacy of anti-phage mechanisms in fermentation production (Dunne et al., 2018).

Non-pathogenic members of the genus *Corynebacterium,* especially *Corynebacterium glutamicum* and *Brevibacterium flavum,* are Gram-positive bacteria widely used as industrial producers in many biotechnological processes (Hermann, 2003). Almost all *Corynebacterium* species are characterized by a complex cell wall architecture: the plasma membrane is covered by a peptidoglycan layer, which is covalently linked to arabinogalactan. Bound to this is an outer layer of mycolic acids. The outer surface components are composed of free polysaccharides, glycolipids, and proteins (Burkovski, 2013).

Corynephage BFK20 causes lysis of the L-lysine producer *Brevibacterium flavum* CCM 251 and morphologically belongs to an unclassified *Siphoviridae* taxonomic group (Koptides et al., 1992). Several corynephages have been isolated from corynebacteria, but most of them have not been characterized in detail. The entire double-stranded DNA genome of bacteriophage BFK20 was sequenced and analysed by Bukovska et al. (2006). Previously, the host range and the adsorption of BFK20 to the cells of selected corynebacterial strains were tested by Halgasova et al. (2005). In this study, BFK20 resistant mutants of *B. flavum* CCM 251 were identified from an EZ-Tn5 transposon insertional library of the *B. flavum* parent strain and phage receptor related genes were identified. The receptors specific for corynephages have not been studied in detail to date. The present study examines adsorption of phage BFK20 on its host bacteria thereby contributing to understanding the phage infection process in corynebacteria.

2. Materials and methods

2.1. Bacteria, bacteriophage, plasmid and growth conditions

The industrial strain *Brevibacterium flavum* CCM 251 (hse^-, aec^r) was used for propagation of phage BFK20 virions (Koptides et al., 1992), chromosomal DNA isolation, and plasmid transformation and isolation. The strain *Corynebacterium glutamicum* RM3 (Schäfer et al., 1990) was used for plasmid transformation and isolation. An EZ-Tn5™ < KAN-2 > Tnp Transposome™ Kit (Epicentre Technologies, Illumina, USA) was used to prepare the *B. flavum* CCM 251 transposon insertion library. The pJUP06 plasmid (Halgasova et al., 2002) was used to express the target genes in the corresponding mutants during the complementation experiments. Bacteria were cultivated in Luria-Bertani (LB) medium at 30 °C. LBHIS medium (van der Rest et al., 1999) was used for growing corynebacterial cells after electroporation. When required, antibiotics were added to the growth media at the following concentrations: 5 or 100 μg/ml kanamycin and/or 10 μg/ml chloramphenicol. The bacterial stocks of the phage resistant mutants were stored in 20% (v/v) glycerol (final concentration) at −80 °C.

2.2. Bacteriophage propagation and assays

Preparation of the BFK20 phage stock and determination of the phage titre were performed as previously described by Halgasova et al. (2005) with one modification: the phage stock was prepared from *B. flavum* CCM 251 using a multiplicity of infection (MOI) of five.

2.3. DNA isolation

The chromosomal DNA from corynebacterial strains was isolated using a Wizard Genomic DNA Purification Kit (Promega, USA) or a Higher Purity™ Bacterial Genomic DNA isolation kit (Canvax, Spain). Corynebacteria plasmid DNAs were isolated according to Santamaria et al. (1984) or using the QIAamp DNA Mini Kit (Qiagen, Germany), with some modifications. In both procedures, lysozyme (15 mg/ml final concentration) was added to the resuspension solution followed by an additional incubation for two hours at 37 °C.

2.4. Electroporation

Competent cells used to construct the *B. flavum* transposon insertional library were prepared according to Oram et al. (2002) with some modifications. Briefly, a single colony was inoculated from a fresh plate into 2 ml of heart infusion broth (Difco, USA) with 0.2% Tween 80 (BHITW), and the cells were grown overnight at 30 °C. The overnight culture was inoculated into 100 ml BHITW supplemented with 2% (w/v) glycine and 15% (w/v) sucrose to give an optical density at 570 nm (OD_{570}) of 0.1. The cells were grown at 30 °C to an OD_{570} of 0.5–0.7. The culture was chilled on ice for 15 min. The cells were then harvested by centrifugation at 4,000 × *g* for 15 min at 4 °C, washed twice with 50 ml ice-cold 15% (v/v) sterile glycerol, and finally resuspended in 0.5 ml 15% (v/v) sterile glycerol. Competent cells used in the complementation experiments were prepared according to van der Rest et al. (1999). Media for preparing competent cells from phage resistant mutants were supplemented with 100 μg/ml kanamycin. The competent cells were either immediately used for electroporation or were frozen in 100 μl aliquots in liquid nitrogen and stored at −80 °C. Electroporation of 100 μl corynebacterial competent cells with 0.1–1.0 μg DNAs was performed in an electroporation cuvette (0.1 cm gap, Bio-Rad, USA) using a gene pulser apparatus (Bio-Rad, USA) with parameters 400 Ω, 1.5 kV, 25 μF. After electroporation we followed the transformation protocols of van der Rest et al. (1999) or Oram et al.

(2002). The cells were incubated for 2 h at 30 °C and aliquots were plated on solid LBHIS medium supplemented with kanamycin and/or chloramphenicol.

2.5. Isolation of B. flavum phage resistant mutants

The EZ-Tn5 transposon insertion library of *B. flavum* was constructed using an EZ-Tn5™ < KAN-2 > Tnp Transposome™ kit (Epicentre Biotechnologies, Illumina, USA). The electroporation of *B. flavum* competent cells (a transformation efficiency of 10^5 cfu/ml) with 20 ng/µl EZ-Tn5 was performed with the parameters given in *2.4*. After electroporation, the samples were immediately transferred to 1 ml of BHI medium with 1% (w/v) glucose, incubated for 3 h at 30 °C and plated on solid LBHIS medium supplemented with 5 µg/ml kanamycin. A collection of nearly 20,000 kanamycin resistant transformants were grown and the colonies from one plate were stripped into 2 ml LB medium and resuspended. The cell suspension was inoculated into 20 ml LB medium to give an OD_{570} of 0.1 and incubated with BFK20 phage stock (MOI of 10) for 4 h at 30 °C on an orbital shaker to enrich the number of phage resistant mutants. The cells were pelleted by centrifugation, resuspended in 1 ml of LB medium and plated in aliquots onto LBHIS plates with 100 µg/ml kanamycin. The presence of the kanamycin resistance cassette in the obtained phage resistant mutants was verified by direct colony PCR with specific primers KanF and KanR (Table S1). 2 µl of crude lysates prepared from individual colonies suspended in 100 µl of sterile water and boiled for 10 min were used as DNA templates. The amplification was performed with a DyNAzyme DNA polymerase or DreamTaq DNA polymerase (Thermo Fisher Scientific) on a T-Gradient thermal cycler (Whatman Biometra).

2.6. Growth rate assay for selection of the phage-resistant mutants

The growth rate of the 700 phage resistant mutants was analysed by incubation in 96-well plates. In duplicates, 20 µl from a mutant overnight culture was diluted into 80 µl of LB medium supplemented with 100 µg/ml of kanamycin and 10 mM $MgSO_4$. At the beginning of incubation, 100 µl BFK20 stock was added to one of the mutant cultures at a MOI of 20 and 100 µl LB medium was added to the parallel sample. *B. flavum* CCM 251 and *B. flavum* CCM 251 with phage (MOI of 20) were used as positive controls. The negative controls were LB medium, LB medium with 100 µg/ml kanamycin and 10 mM $MgSO_4$, and phage BFK20 stock solution (all in 200 µl volumes). The plates were incubated at 30 °C with shaking for 24 h and OD_{600} readings were taken every 2 h using a Synergy HT Multi-Mode Microplate Reader (Biotek). The measurements were repeated at least three times for each of the 700 mutants.

The growth rate of forty selected phage resistant mutants was analysed by cultivation in flasks in 50 ml volumes. The overnight cultures of mutant strains were inoculated in parallel into 50 ml LB medium each supplemented with 100 µg/ml kanamycin and 10 mM $MgSO_4$ to give an OD_{570} of 0.1. At the beginning of cultivation, BFK20 was added to one mutant culture at a MOI of 20. The samples were incubated at 30 °C with shaking and OD_{570} readings were taken after 2, 4, 6, 8, 10 and 24 h. The *B. flavum* and *B. flavum* with BFK20 positive controls were measured simultaneously. All growth rate experiments were repeated at least three times.

2.7. Identification of the disrupted genes by single primer PCR

Single primer (SP) PCR, which uses only one primer, was used to identify the transposon EZ-Tn5 insertion site in the genomes of the phage resistant mutants. Briefly, genomic DNA from the forty phage resistant mutants was isolated with an isolation kit and 200 ng was used as a template DNA. The amplification was performed with one of the specific primers Tn5-F2, TN5-F3 or Tn5-R2 (Table S1). The reaction involved three steps: after initial denaturation at 94 °C for 2 min, the first step included 29 cycles of denaturation (94 °C for 15 s), annealing (60 °C for 30 s) and extension (72 °C for 2 min); the second step included 29 cycles of denaturation (94 °C for 15 s), annealing (40 °C for 30 s) and extension (72 °C for 2 min); the final step included 29 cycles of denaturation (94 °C for 15 s), annealing (60 °C for 30 s), extension (72 °C for 2 min); the process finished with a final extension at 72 °C for 7 min. The amplification was performed with a DyNAzyme DNA polymerase or DreamTaq DNA polymerase (Thermo Fisher Scientific, USA) on a T-Gradient thermal cycler (Whatman Biometra, Germany). The PCR products were electrophoresed on a 1% agarose gel and the DNA bands were cut out of the gel and purified with a QIAquick Gel Extraction Kit (Qiagen, Germany). These PCR fragments were sequenced (GATC Biotech, Germany) using sequencing primers KAN-2-FP-1 for the Tn5-F2 and TN5-F3 generated products, and KAN-2-RP-1 for the Tn5-R2 generated products (Table S1). The resulting sequences of these SP PCR products were used as a query to search the Blast non-redundant database using BLASTX and BLASTP (Altschul et al., 1997) and the *Corynebacterium glutamicum* ATCC 13032 genome database (NCBI number BA000036). A Conserved Domain Database (CDD, Marchler-Bauer et al., 2017) search was used to find conserved domains in the proteins.

2.8. Adsorption assay

For the adsorption assay, cultures of corynebacterial strains were grown in 10 ml of LB medium or LB supplemented with an antibiotic. At an OD_{570} of 0.3, $MgSO_4$ was added to a final concentration of 10 mM and the bacterial culture was infected with BFK20 at a MOI of 0.5. Aliquots were removed 0 min and 20 min after phage infection, diluted to 10^{-3} and centrifuged at 14,000 × *g* for 10 min to separate the cells with adsorbed phage from free phage particles. The concentration of free phage particles in the supernatant was determined in titer analysis using the double-layer plate method according to Halgasova et al. (2005). A sample of supernatant with phage but without corynebacteria cells was used as a negative control; *B. flavum* CCM 251 cells infected with phage (MOI of 0.5) were used as a positive control. The adsorption assay for each phage resistant mutant was repeated at least three times.

2.9. Complementation assay

The MFS protein (NCgl2141) and glycosyltransferase (NCgl0321) target genes for the complementation tests were amplified by PCR using specific primers (Table S1) containing a Bsu36I restriction site. 100 ng *B. flavum* genomic DNA was used as a template. Amplification was performed using the Phusion High-Fidelity DNA Polymerase (Thermo Scientific, USA). The amplified PCR products of the target genes were ligated into the pJUP06 corynebacterial vector (Halgasova et al., 2002), digested with Bsu36I and transformed by electroporation into *C. glutamicum*. The resulting plasmids pJUP06-MFS, pJUP06-GT and pJUP06-PGT were isolated and the sequence and orientation of the cloned fragments were verified by sequence analysis (GATC Biotech, Germany) using specific sequencing primers (Table S1). Recombinant plasmids were transformed into their corresponding mutants by electroporation. The sensitivity of the transformants to BFK20 was analysed by growth rate assays in 50 ml volumes (*2.6*) and by adsorption assays (*2.8*). *B. flavum* CCM 251 harbouring pJUP06 was used as a control. All assays were repeated at least three times.

2.10. Efficiency of plating analysis

Efficiency of plating (EOP) was determined according to Kutter (2009). In brief, the cultures of corynebacterial strains were grown overnight in 10 ml of LB medium or LB supplemented with an antibiotic. The phage BFK20 stock of 5×10^8 PFU/ml was serially diluted and 50 µl of relevant phage suspension was mixed with 50 µl culture of *B. flavum* TMs, complemented *B. flavum* TM and host bacterium. After

5 min incubation the samples were poured over an LB plate with 3 ml of soft agar using the double-layer method (Halgasova et al., 2005). For all TM strains a stock dilution 10^7 was used, except the *B. flavum* TM33, where stock dilution was 10^1. The relative EOP was calculated as the ratio of the phage titre (PFU/ml) obtained in each TM and that obtained in the host. Three independent experiments were performed in duplicate.

3. Results

3.1. Identification of the disrupted genes in B. flavum phage resistant mutants

The EZ-Tn5 transposon insertion sites in the genomes of the forty *B. flavum* phage resistant mutants were determined by analysing the PCR fragments produced by SP PCR using specific forward or reverse primers (Table S1). *B. flavum* is considered to be the same species as *C. glutamicum* (Liebl et al., 1991); therefore, the genes disrupted in the *B. flavum* mutants were identified based on their homology to the known genes in the genomic sequence of *C. glutamicum* ATCC 13032 (NC_003450.3). Eight mutants had a disruption in a gene encoding a major facilitator superfamily (MFS) permease (NP_601425.2), and the EZ-Tn5 insertion was found in four different sites (illustrated by the *B. flavum* transposition mutants (TM) TM3, TM7, TM54 and TM67 in Fig. 1a). Nine mutants had disruptions in a gene encoding a ketoglutarate semialdehyde dehydrogenase (ADH) (NP_599699.1); two different insertion sites were found (e.g. *B. flavum* TM11 and TM41 in Fig. 1b). Another eight mutants were disrupted in a gene coding for an ATPase involved in pili and flagella biosynthesis (NP_599554.2); three different insertion sites were found (e.g. *B. flavum* TM1, TM14 and TM20 in Fig. 1c). Only one *B. flavum* mutant, TM33, was identified with a disrupted glycosyltransferase (GT) gene, which is probably involved in cell wall polysaccharides synthesis (NP_599579.1) (Fig. 1d). Table 1 lists the interrupted genes that were identified, their corresponding proteins, ten selected phage resistant mutants, and the sites of transposon insertion in the disrupted protein (mutants with the same insertion sites are not listed). Fig. 1 schematically illustrates the location of the EZ-Tn5 transposon insertion sites in each of the four proteins of these ten selected mutants. Based on the results of similar studies (Cui et al., 2016; Li et al., 2016b), it seemed likely that at least the MFS protein and the glycosyltransferase would have roles in phage receptor synthesis. The remaining fourteen *B. flavum* mutants tested had disruptions in transposase (NCgl1464), acyl-CoA synthetase (NCgl2774) and nucleoside-diphosphate-sugar epimerase (NCgl0317) genes.

3.2. Growth rate assay of selected phage–resistant mutants

The resistance of the forty *B. flavum* transposition mutants to BFK20 infection was analysed by cultivation in 50 ml volumes (section *2.6*; *B. flavum* CCM 251 was used as a control). The resulting growth curves of the ten *B. flavum* transposition mutants listed in Table 1 are plotted in Fig. 2. In all cases, the *B. flavum* CCM 251 control strain showed a typical growth curve up to stationary phase, and the infected culture lysed within 6 h of infection. The growth curves of the *B. flavum* mutants TM3, TM7, TM54 and TM67, which have a disrupted MFS protein, are shown in Fig. 2a. Their growth rates in the absence of phage were similar to the *B. flavum* control strain, though with higher absorbance. After phage infection, however, the mutant cultures showed no signs of lysis up to 24 h, and their growth curves remained similar to that of the *B. flavum* control strain without phage. This clearly shows that the modified strains are resistant to BFK20 infection. The other *B. flavum* transposition mutants behaved similarly (Fig. 2b–d).

3.3. Adsorption rate assay of the phage resistant mutants

The ability of these phage-resistant mutants to adsorb phages was tested, with the wild-type *B. flavum* CCM 251 used as a control. The results of the adsorption assay for the ten mutants listed in Table 1 together with the control strain are shown in Fig. 3. After 20 min 60% of the BFK20 virions had adsorbed onto the *B. flavum* control cells. Adsorption on the phage-resistant mutants, however, ranged from 6.2 to 20.0%. A phage adsorption ability below 20% clearly indicated that these mutants had impaired phage receptors. The results for each mutant represent an average of at least three measurements.

3.4. Complementation of the phage resistant mutants

In order to verify whether the interrupted genes were in fact responsible for the phage-resistance phenotypes, the target genes of those proteins most likely to be directly involved in phage receptor synthesis, the MFS protein and the glycosyltransferase (with and without their natural promoters) were cloned into the corynebacterial vector pJUP06 (Halgasova et al., 2002) to create the recombinant plasmids pJUP06-MFS, pJUP06-GT and pJUP06-PGT (Fig. 4a–c). Although we tested more than a hundred transformants, we were not able to obtain constructs of a pJUP06-PMFS plasmid. The orientation of the fragments encoding MFS, GT and PGT in pJUP06 was determined by sequencing using the sequencing primers given in Table S1. Recombinant plasmid pJUP06-MFS was transformed by electroporation into *B. flavum* TM3 and TM7 cells, and plasmids pJUP06-GT and pJUP06-PGT were electroporated into *B. flavum* TM33 cells. The resulting complemented strains, *B. flavum* TM3_MFS, TM7_MFS, TM33_GT and TM33_PGT, were tested for their sensitivity to BFK20 infection; *B. flavum* CCM 251 harbouring plasmid pJUP06 was used as a control. The control strain, mutants and complemented mutants were cultivated in 50 ml volumes with and without phage, and the resulting resulted growth curves are shown in Fig. 5. Cells of the control strain without phage showed a typical growth curve up to stationary phase and lysed after phage infection. As before, *B. flavum* TM3, *B. flavum* TM7 and *B. flavum* TM33 showed typical growth curves regardless of the presence of BFK20, thereby confirming their resistance to infection (Fig. 5a–d). The complemented mutants *B. flavum* TM3_MFS, *B. flavum* TM7_MFS, *B. flavum* TM33_GT and *B. flavum* TM33_PGT also displayed a typical growth curve in the absence of BFK20, but during cultivation in the presence of the phage, these cultures revealed sensitivity to BFK20 infection: the optical density of *B. flavum* TM3_MFS and *B. flavum* TM7_MFS showed no increase up to 8 h after infection (Fig. 5a,b) and the cultures of *B. flavum* TM33_GT and *B. flavum* TM33_PGT did not grow within the 10 h of cultivation (Fig. 5c,d).

The phage adsorption ability of these four complemented mutants was also tested. *B. flavum* TM3_MFS and *B. flavum* TM7_MFS cells displayed similar adsorption rates, 46.5% and 41.0%, respectively, as the control strain (45.7%; Fig. 6), while the *B. flavum* TM33_GT and *B. flavum* TM33_PGT cells exhibited an even higher adsorption ability (50.7% and 51.3%, respectively). Their levels were nearly the same as those of the untransformed *B. flavum* host strain (58.9%) (Fig. 6). It therefore appears that sensitivity to BFK20 was restored to the *B. flavum* phage resistant mutants TM3, TM7 and TM33 by the trans-complementation of the intact genes of the MFS and GT proteins. Consequently, disruption of the MFS protein and GT genes was very likely responsible for the phage-resistant phenotype exhibited by these mutants, and that these genes probably involved in phage receptor synthesis.

3.5. Efficiency of plating analysis of the phage resistant mutants

The relative EOP was determined for ten *B. flavum* TMs and four complemented *B. flavum* TMs (Fig. 7). EOP value of the host strain *B. flavum* CCM 251 was considered 1. The results indicated reduction of BFK20 infection efficiency for all tested *B. flavum* TMs. The most significant decrease of BFK20 infection was demonstrated for *B. flavum* TM33 with EOP value 1.59×10^{-7}. This result indicated that phage

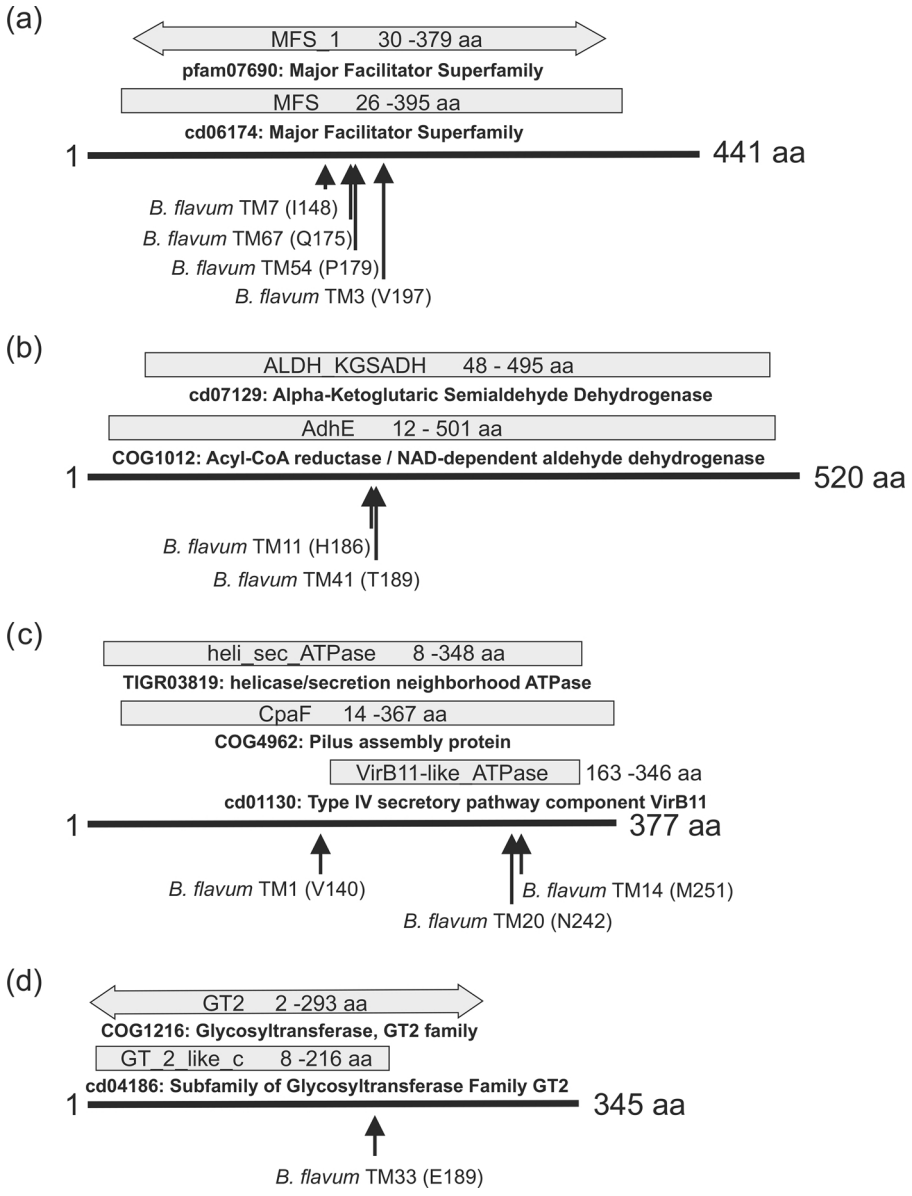

Fig. 1. Schematics of the protein products of four genes interrupted by insertion of the EZ-Tn5 transposon *B. flavum* TMs. Specific sequence hits identified by searches against the CDD are shown. The arrows mark the gene interruption sites of ten selected *B. flavum* TMs. The protein products of the interrupted genes are a) a MFS transporter, b) a ketoglutarate semialdehyde dehydrogenase, c) an ATPase, d) a glycosyltransferase.

Table 1
EZ-Tn5 transposon insertion sites in the *B. flavum* genome.

C. glutamicum ATCC 13032 (NC_003450.3) gene corresponding to disrupted *B. flavum* gene Gene ID / Locus tag	Encoded protein (cds)	Protein length (aa)	Selected phage resistant mutants of *B. flavum* CCM 251	Site of EZ-Tn5 insertion in disrupted protein (aa)
Gene ID 1020174 / NCgl2141	MFS protein - major facilitator superfamily permease (NP_601425.2)	441	*B. flavum* TM3	V197
			B. flavum TM7	I148
			B. flavum TM54	P179
			B. flavum TM67	Q175
Gene ID 1021211 / NCgl0437	ketoglutarate semialdehyde dehydrogenase (NP_599699.1)	520	*B. flavum* TM11	H186
			B. flavum TM41	T189
Gene ID 1021365 / NCgl0297	ATPase involved in pili and flagella biosynthesis (NP_599554.2)	377	*B. flavum* TM1	V140
			B. flavum TM14	M251
			B. flavum TM20	N242
Gene ID 1021368 / NCgl0321	Glycosyltransferase, GT2 family (NP_599579.1)	345	*B. flavum* TM33	E189

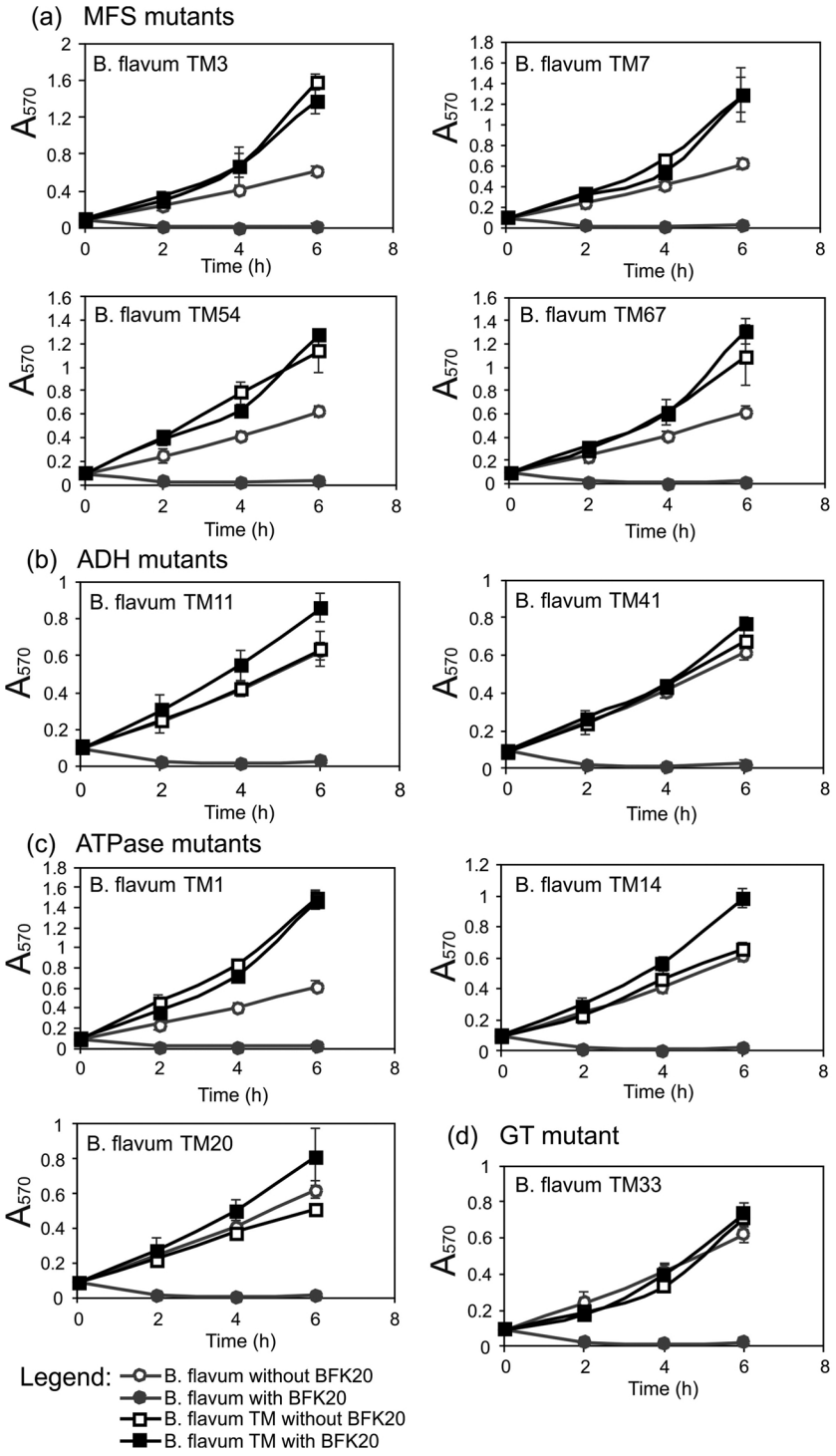

Fig. 2. Growth rate assay of the *B. flavum* TMs. *B. flavum* TMs and *B. flavum* CCM 251 were infected with BFK20 at a MOI of 20. Growth curves of *B. flavum* TMs with interruptions in genes encoding an MFS transporter, a ketoglutarate semialdehyde dehydrogenase, an ATPase and a glycosyltransferase are shown in a), b), c) and d), respectively. Curves for selected *B. flavum* TMs and the *B. flavum* CCM 251 control without BFK20 are shown in parallel. The values at each time point represent the average of three independent experiments ± SD.

receptors of TM33 were significantly impaired. A much smaller decrease of BFK20 infection could be observed for other transposition mutants, they demonstrated EOP values ranging from 0.11 (*B. flavum* TM7) to 0.593 (*B. flavum* TM11). BFK20 infection ability was restored nearly to value of the host strain in complemented *B. flavum* TM33_GT (with EOP value of 0.82), *B. flavum* TM33_PGT (EOP of 0.92), *B. flavum* TM3_MFS (EOP of 0.82) and *B. flavum* TM7_MFS (EOP of 0.83). The results of EOP gave much more a quantitative analysis of phage infection impairment obtained especially for mutant TM33 compare to the other mutants. This suggested significant role of glycosyltransferase gene in BFK20 receptor synthesis.

4. Discussion

Bacteriophage adsorption onto the host cell is essential for initiating infection of the bacteria. This process depends on the recognition of

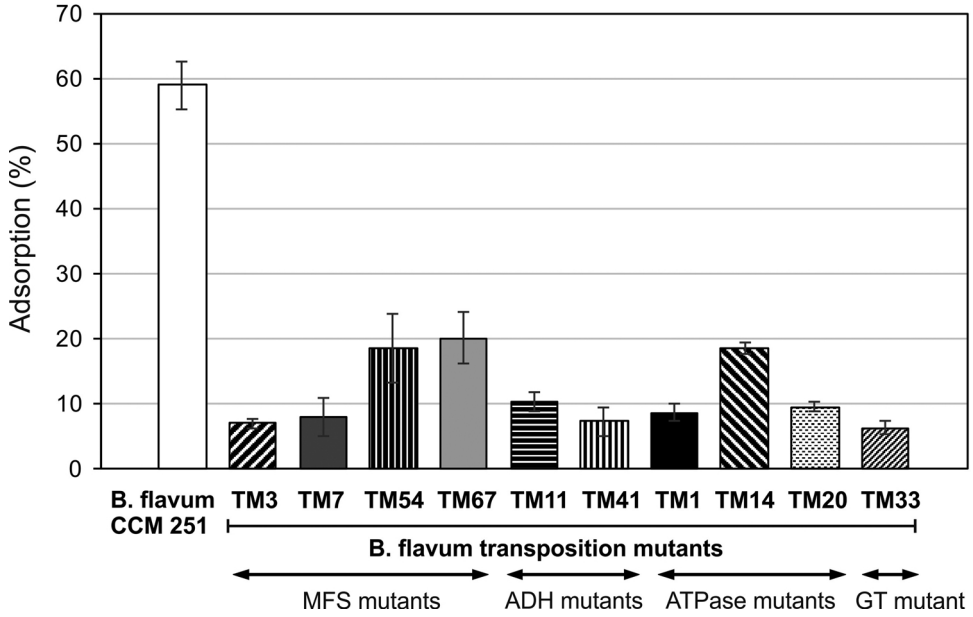

Fig. 3. Adsorption assay. Adsorption of phage BFK20 at a MOI of 0.5 to *B. flavum* MFS mutants, ADH mutants, ATPase mutants and a GT mutant 20 min after infection shown together with adsorption to the *B. flavum* CCM 251 control. The values represent the average of three independent experiments ± SD.

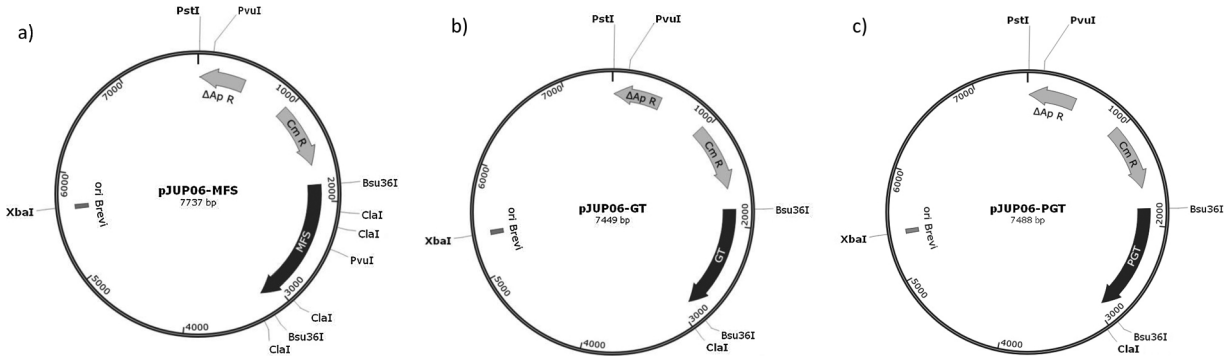

Fig. 4. Schemes of the recombinant plasmids a) pJUP06-MFS, b) pJUP06-GT and c) pJUP06-PGT. Abbreviations: MFS transporter gene (MFS), glycosyltransferase (GT), glycosyltransferase with promotor (PGT), a brevibacteria origin of replication (*ori* Brevi), chloramphenicol resistance selection marker (Cm R), truncated ampicillin resistance selection marker (ΔApR) (19–490 bp). The positions of the cloning restriction sites (*Bsu*36I) are indicated.

phage-specific receptors on the bacterial cell surface (Letarov and Kulikov, 2017). The nature and location of the host cell receptors recognized by a given bacteriophage varies depending on the phage and the host (Bertozzi Silva et al., 2016). According to the Phage Receptor Database (PhReD, available at www.ualberta.ca/phred), nearly every cell surface structure can serve as a phage receptor (Bertozzi Silva et al., 2016). Up to date only a small number of receptors have been identified for phages infecting Gram-positive bacteria. This is due in part to their complex outer structure and, in part, to the limited research done on phages that target Gram-positive bacteria generally (Dowah and Clokie, 2018).

Corynebacteria are Gram-positive aerobic bacteria of diverse species. Many non-pathogenic corynebacteria are of medical, veterinary or biotechnological importance (Oliveira et al., 2017). Members of the genera *Corynebacterium* and *Brevibacterium* are widely used as industrial producers in many biotechnological processes, especially in the production of essential amino acids (Hermann, 2003). The existence of lytic and temperate phages in corynebacteria has been known for many years (Kato et al., 1984; Patek et al., 1985). Most of the isolated corynephages are temperate, have a very narrow host range, and mostly belong morphologically to the *Siphoviridae* taxonomic group (Ackermann, 2009). Despite the industrial and medical importance of corynebacteria, the genomes of only five corynephages have been sequenced and analysed to date (Bukovska et al., 2006; Chen et al., 2008; Lobanova et al., 2017; Yomantas et al., 2018).

Corynephage BFK20 is a lytic phage of *B. flavum* CCM 251, an industrial L-lysine producer, and it was the first corynephage to have its genome completely sequenced and analysed in detail (Bukovska et al., 2006, EMBL accession no. AJ278322). Previously, we tested the adsorption of BFK20 to the corynebacterial cell surface. We observed strong adsorption, exceeding 75%, for *B. flavum* strains, minor adsorption for *B. lactofermentum* (13%) and no adsorption for *C. glutamicum* (Halgasova et al., 2005).

The current work focuses on identifying of protein-coding genes of the host bacteria *B. flavum* CCM 251 involved in BFK20 adsorption. According to the literature, the host genes most frequently found to be involved in phage recognition and adsorption are either associated in some way with the stages of phage infection, or participate in receptor synthesis (Bertozzi Silva et al., 2016). Based on our results, we concluded, that identified protein-coding genes are probably involved in the syntheses of the receptors required for BFK20 infection. These proteins were identified by preparing an EZ-Tn5 transposon insertional library from the host strain and we screened the resulting mutants for BFK20 phage resistance. Sequence analysis against the *C. glutamicum* ATCC 13032 genomic sequence (EMBL Acc. No. BA00036), showed that seven protein-coding genes had been disrupted. We decided to study

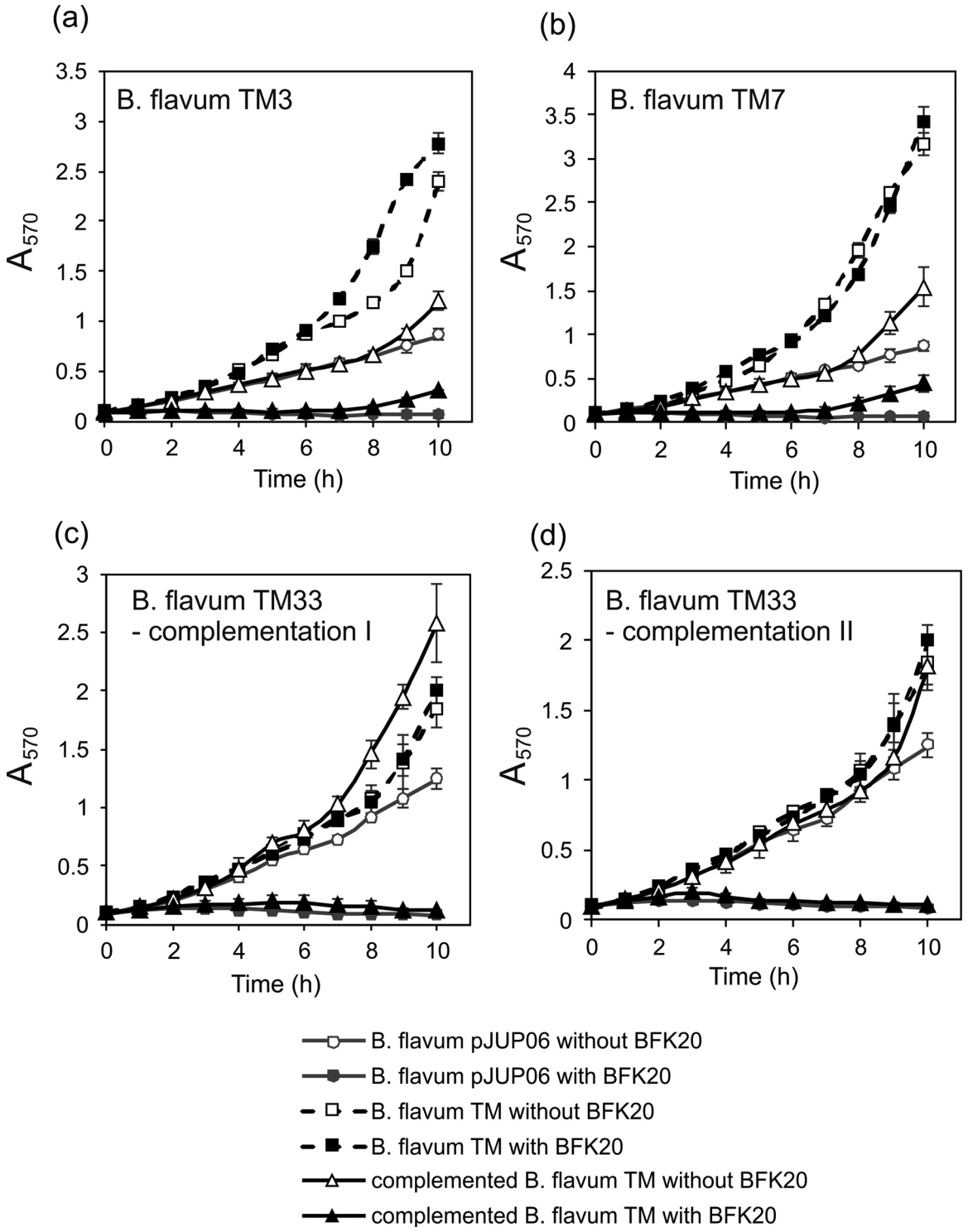

Fig. 5. Growth rate assay of *B. flavum* TMs after complementation. a) growth curves of *B. flavum* TM3_MFS, *B. flavum* TM3 and *B. flavum* with pJUP06 (a control), infected with BFK20 at a MOI of 20; b) *B. flavum* TM7_MFS, *B. flavum* TM7 and *B. flavum* with pJUP06 (a control), infected with BFK20 at a MOI of 20; c) *B. flavum* TM33_GT, *B. flavum* TM33 and *B. flavum* with pJUP06 (a control), infected with BFK20 at a MOI of 20; d) *B. flavum* TM33_PGT, *B. flavum* TM33 and *B. flavum* with pJUP06 (a control), infected with BFK20 at a MOI of 20. For the selected *B. flavum* TMs and the *B. flavum* with pJUP06 control, samples with and without BFK20 are shown in parallel. The values at each time point represent the average of three independent experiments ± SD.

only four of these in detail: an MFS protein (NP_601425.2), a ketoglutarate semialdehyde dehydrogenase (ADH) (NP_599699.1), an ATPase (NP_599554.2) and a glycosyltransferase (GT) (NP_599579.1) (Fig. 1). The remaining *B. flavum* mutants with disruptions in transposase (NP_600737.1), acyl-CoA synthetase (NP_602064.1) and nucleoside-diphosphate-sugar epimerase (NP_599575.1) will be tested in next experiments. Localisation some of identified genes on chromosome (NC_003450.3) revealed their position in a cluster, specifically genes coding a glycosyltransferase (GT) (NP_599579.1) and nucleoside-diphosphate-sugar epimerase (NP_599575.1). Moreover, using bioinformatics (Overbeek et al., 2014) a nucleoside-diphosphate-sugar epimerase was identified as a protein potentially involved in biosynthesis

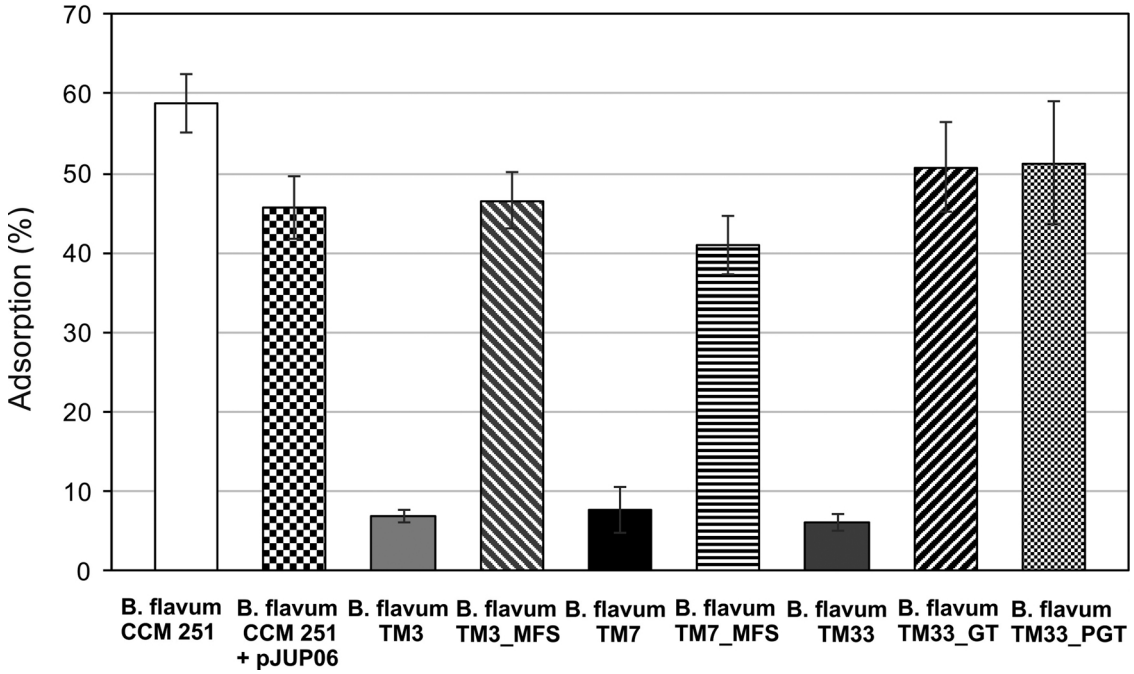

Fig. 6. Adsorption assay. Adsorption of phage BFK20 at a MOI of 0.5 to *B. flavum* TM3_MFS, *B. flavum* TM7_MFS, *B. flavum* TM33_GT and *B. flavum* TM33_PGT 20 min after infection shown in parallel with adsorption to *B. flavum* TM3, *B. flavum* TM7 and *B. flavum* TM33. *B. flavum* CCM 251 and *B. flavum* CCM 251 with pJUP06 served as controls. The values represent the average of three independent experiments ± SD.

of rhamnose containing glycan.

The rhamnose cell wall polysaccharides (RhaCWP) are important phage receptors for many species (Bertozzi Silva et al., 2016). In bacteria, RhaCWP biosynthesis genes are often located in clusters encoding between 12 and 25 genes with functions such as glycosyltransferases, polysaccharide biosynthesis proteins, rhamnose biosynthesis proteins and putative transport molecules. Biosynthesis of RhaCWP is likely initiated on the inside of the cytoplasmic membrane on a lipid carrier through the step-wise addition of monosaccharides by specific glycosyltransferases. In next the structure is transported across the membrane and attached to peptidoglycan. In some bacteria, the transport of big sugar complexes across cell membranes requires complex consisting of a permease protein and an ATP-binding protein (Mistou et al., 2016). We hypothesize that the identified glycosyltransferase is likely involved in the synthesis of essential saccharide components of the *B. flavum* cell envelope and MFS permease and ATPase are probably involved in transport of the saccharide complexes across cell membranes.

Generally, MFS proteins belong to one of the biggest families of secondary active membrane transporters. Membrane proteins with transport function serve as receptors for phages infecting Gram-negative bacteria. GTs are frequently involved in the synthesis of glycolipids, peptidoglycan (PG), and of lipooligosaccharides (LOS), which are essential components of the cell envelope. Glycolipids and LOS can be suitable targets for the binding of phage virions in Gram-negative bacteria. For phages that infect Gram-positive bacteria, peptidoglycan is an important phage receptor, since it is a major polymer on bacterial surfaces.

We tested the sensitivity of these transposition mutants to BFK20

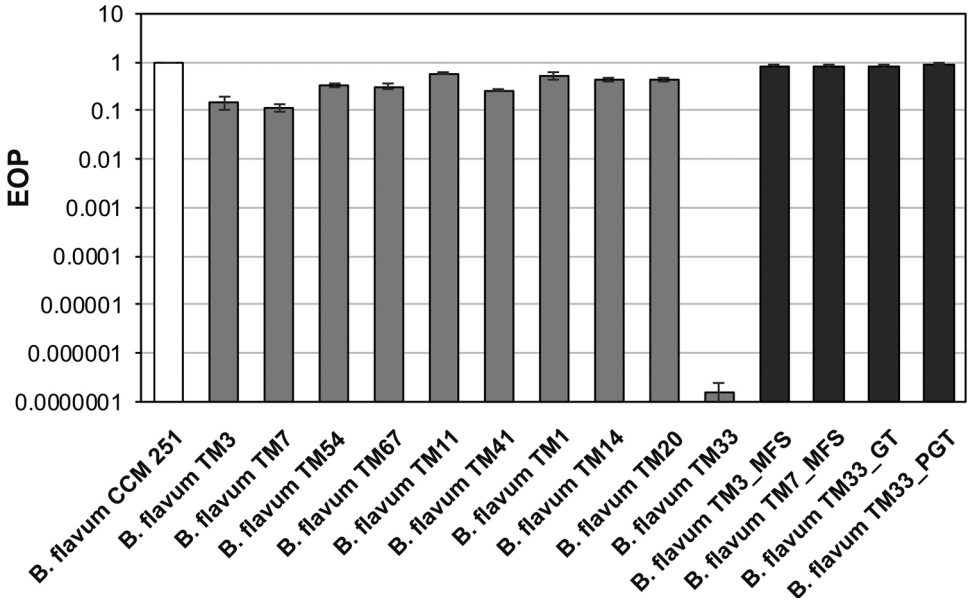

Fig. 7. Efficiency of plating of *B. flavum* TM strains relative to the host strain *B. flavum* CCM 251. The EOP was evaluated for ten *B. flavum* TM (light grey columns) and four complemented *B. flavum* TM (dark grey columns). *B. flavum* CCM 251 served as a reference strain (white column). The values represent the average of three independent experiments ± SD.

phage infection by cultivation and the ability of the phage to adsorb to their cell walls; the results from only ten selected, representative *B. flavum* transposition mutants are described here. None of the ten mutants showed any signs of lysis after cultivation with BFK20, suggesting that the disruption of these genes are responsible for producing the phage resistance phenotype (Fig. 2). That this resistance arises from the corruption of the phage receptors on the bacterial cell surface was confirmed by testing the ability of phages to adsorb to these cells. These mutants had significantly reduced adsorption rates of under 20.0% as compared to the 60% of the *B. flavum* parent strain (Fig. 3). To further validate that the disrupted genes were responsible for the phage resistance phenotype we carried out complementation assays for two of these genes. The intact MFS protein and GT genes on a pJUP06 plasmid were transformed into the corresponding *B. flavum* TM3, TM7 and TM33 mutants and the same cultivation and adsorption assays were carried out. The results showed that the sensitivity of the mutants to phage BFK20 had been restored: the *B. flavum* TM3_MFS, and TM7_MFS cultures did not grow within 8 h cultivation with phage, the *B. flavum* TM33_GT and TM33_PGT cultures did not grow within 10 h (Fig. 5). The complementation also restored the ability of BFK20 virions to adsorb to these cells as well (Fig. 6). The complemented *B. flavum* TM33_GT and TM33_PGT mutant adsorption reached 50.7 and 51.3%, respectively, similar to the 58.9% adsorption of the wild-type *B. flavum* host strain. The results of EOP analysis (Fig. 7) confirmed the results of growth and adsorption assays. The EOP was reduced for all *B. flavum* transposition mutants, most significantly for *B. flavum* TM33. The EOP of TM strains after complementation were comparable with value of *B. flavum* host strain.

Recently, studies similar to ours were carried out on *Pseudomonas aeruginosa* and its C11 (Cui et al., 2016) and K5 (Li et al., 2016b) phages. Cui et al. (2016) prepared a library of *P. aeruginosa* mutants using a Tn5G transposon and screened the resulting phage resistant mutants. They identified seven disrupted *P. aeruginosa* genes, including *wbpR,* which encodes a glycosyltransferase, and *PA1993*, which encodes a probable MFS transporter. The glycosyltransferase was verified to be responsible for the phage-resistant phenotype by complementation, but the MFS transporter was not. Since glycosyltransferase is involved in LPS synthesis in Gram-negative bacteria, the authors concluded that LPS was probably the final receptor for phage C11 binding. In the study by Li et al. (2016b), thirteen phage-resistant mutants were isolated from a Tn5G transposon library of *P. aeruginosa*. They also found that a glycosyltransferase-encoding gene, *wapH,* and probably transport protein-encoding gene, *PA4334,* were among the disrupted genes that appeared to confer phage resistance. The complementation experiments again showed that complementation with *wapH* successfully restored sensitivity to phage K5 in the corresponding mutants and the transporter protein was not confirmed. Although *P. aeruginosa* is Gram-negative and phages C11 and K5 are *Myoviridae* viruses, these results can still be taken to partially corroborate our findings. In our case, however, we were able to confirm that the glycosyltransferase and also the MFS protein are both potentially involved in phage receptor synthesis in *B. flavum*.

Other studies, using transposon library, similar to ours have also been carried out in various systems, but mostly on phages infecting Gram-negative bacteria (Filippov et al., 2011; Zhang et al., 2009; Shin et al., 2012; Xu et al., 2013); less research has been done on phages specific to Gram-positive bacteria (Chapot-Chartier, 2014; Cvirkaite-Krupovic et al., 2015).

Our results, therefore, contribute to the comparatively small list of identified receptors for those phages infecting Gram-positive bacteria. This is also the first such study to identify some of the genes and proteins involved in the synthesis of the phage receptors of any corynephage. The adsorption mechanism during the early stages of phage infection in corynebacteria has not been studied in detail to date. In future studies, we plan to examine the complementation ability of the other *B. flavum* receptor-related genes identified here and to verify their contribution to phage-host interactions.

5. Conclusion

In conclusion, four *B. flavum* CCM 251 genes coding for an MFS protein, an ADH, an ATPase and a glycosyltransferase that are likely to be involved in the syntheses of the receptors required for BFK20 infections were identified. The phage resistant phenotype of ten selected *B. flavum* mutants was confirmed by cultivation with phage, by phage adsorption and efficiency of plating assays. Complementation experiments demonstrated that intact MFS protein and GT genes provided *in trans* on plasmid pJUP06 successfully restored sensitivity to BFK20 to the corresponding mutants. The result of efficiency of plating on *B.flavum* strain with disrupted GT gene showed the significantly reduced ability of BFK20 infection. We assume that the potential target receptors for phage BFK20 binding could be some of the components of the corynebacterial cell envelope, most probably cell wall polysaccharides. The further studies of the target receptors for phage BFK20 binding are needed to determine this presumption.

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Declaration of Competing Interest

All authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.197775.

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