Glycosyl hydrolases from *Bifidobacterium adolescentis* DSM20083

Their role in the metabolism and synthesis of oligosaccharides

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ABSTRACT

Key words: *Bifidobacterium adolescentis*, prebiotic, glycosyl hydrolases, transglycosylation, non-digestible oligosaccharides.

Nowadays, in the food industry there is increasing interest in positively influencing the intestinal microbiota through the diet by the use of probiotics and/or prebiotics. Especially the number of microorganisms belonging to the genera *Bifidobacterium* and *Lactobacillus* should be increased, because it is claimed that they have health promoting effects for the host. Bifidobacteria play an important role in carbohydrate fermentation in the colon and *Bifidobacterium adolescentis* is one of the main species of bifidobacteria in human adults.

To obtain more information about their carbohydrate modifying enzymes, a genomic library of *B. adolescentis* DSM20083 was constructed and screened for glycosyl hydrolases. Also the possibilities of the use of heterologously expressed glycosyl hydrolases for the synthesis of tailor made oligosaccharides, which might be used as prebiotic, were studied. Genes encoding for an α-galactosidase, two α-glucosidases, a sucrose phosphorylase, and an arabinoxylan arabinofuranohydrolase-D3 were identified. The genes were cloned and expressed in *Escherichia coli* and the proteins were biochemical characterized. The 3D structure of sucrose phosphorylase was elucidated, which is the first structure of a phosphate-dependent enzyme from the glycoside hydrolase family 13. All enzymes were retaining except for arabinoxylan arabinofuranohydrolase-D3, which was inverting. All retaining enzymes were able to synthesize oligosaccharides. The recombinant sucrose phosphorylase synthesized a novel non-reducing dimer, Glc*p*(1→1)β-Ara*f*, which structure was elucidated by NMR. Site directed mutagenesis was applied to improve the synthesizing properties of the α-galactosidase. Furthermore an overview is presented of glycosyl hydrolases of bifidobacteria and their relation to prebiotics.

CONTENTS

Chapter 1

General Introduction

INTRODUCTION

Nowadays, consumers are becoming more and more aware of the link between health, nutrition, and diet. In Japan there is a traditional belief that health is dependent on food and the term functional foods as marketing term was initiated in Japan in the late 1980s (Farnworth 2002; Shah 2001; Stanton et al. 2001). Functional foods obtained a legal status and specific labeling benefits in 1991 and are known as FOSHU (FOods for Specified Health Use; Sanders 1998). The definition of functional food is 'foods that contain some health-promoting component(s) beyond traditional nutrients' (Berner and O'Donnell 1998). The functional food market in Europe, the U.S., Japan, and Australia has been valued at $5.7 billion in 2000 (Hilliam 2000) and is still rapidly increasing. Reasons why functional food are attractive to consumers are (i) consumers want to prevent, rather than cure, disease; (ii) rise of medical costs; (iii) consumers are more aware of link between health and nutrition; (iv) aging population; (v) want to counteract the perceived increase of environmental hazards from pollution, microbes and chemicals in air, water, and food; (vi) increased scientific evidence for efficacy (Sanders 1998). Dairy products are significant players in the functional food market; for example, they are estimated to account for approximately 60% of the European market (Young 2000).

Diary foods are one of the major food groups contributing to a balanced diet. The composition of the diet is one of the parameters, which can influence the gut microbiota balance (Shortt et al. 2004). It is assumed that the increase of high numbers of good bacteria in the gut, belonging to the genera *Lactobacillus* and *Bifidobacterium*, improve health and well being of the host (O'Sullivan 2001; Tomasik and Tomasik 2003). Consumers are already familiar with diary products and, therefore, it is possible to use fermented dairy products to increase the number of good bacteria in humans (Shortt et al. 2004). Examples are probiotics and prebiotics, which are used to influence the gut microbiota composition in a positive way (Hammes and Hertel 2002).

PROBIOTIC, PREBIOTIC, AND SYNBIOTIC

The word probiotic is translated from the Greek meaning 'for live' and the definition of probiotic is 'preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the micro flora (by implantation or colonization) in a compartment of the host and by that exert

beneficial health effects in this host' (Schrezenmeir and De Vrese 2001). The intake of bacterial drinks by humans is over 2000 years old, although Metchnikoff reported the first introduction on a scientific basis in 1908. He observed the long life of Bulgarian peasants who consumed fermented milk foods (Metchnikoff 1907; 1908). Nowadays, the production and consumption of probiotic products are rapidly increasing in Europe. Table 1 gives an overview of the proposed criteria for microorganisms to be used as probiotic. The most common microorganisms used are from the genera *Lactobacillus* and *Bifidobacterium* (Farnworth 2001; Isolauri et al. 2004; O'Sullivan 2001). Potential health and nutritional benefits of these probiotic bacteria are improved digestibility, nutritional value, and lactose utilization but also antagonistic action towards enteric pathogens, colonization in the gut, anticarcinogenic effect, hypochlolesterolaemic action, immune modulation, vitamin production, and as vaccine vehicle (Gomes and Malcata 1999; Shortt et al. 2004). An overview of commercially marketed formulations and products of probiotics is given by Gomes and Malcata (1999) and Kaur et al. (2002).

TABLE 1: Proposed criteria for microorganisms used in probiotics[a]

1	Microorganism of human origin
2	Resistance to acid conditions of stomach, bile, and digestive enzymes normally found in the human gastrointestinal tract
3	Resistance to destruction by technical processing
4	Ability to colonize human intestine
5	Safe for human consumption
6	Scientifically proven efficacy

[a] Farnworth (2001), O'Sullivan (2001), and Teitelbaum and Walker (2002)

It is assumed that the minimal dose of probiotic bacteria should be 10^7 CFU/g food to obtain the desired effect, because the bacteria must overcome a number of physical and chemical barriers like gastric juice and bile acid in the gastrointestinal tract (GIT). The result can be that the probiotic bacteria reach the colon in a sort of stressed state that will negatively affect the ability to colonize the colon. On the other hand, there is no proof to date of a need for colonization, and following dietary intake most probiotics reside only transiently (Isolauri et al. 2004). Several authors have

reviewed the effect of probiotic bacteria on gastrointestinal diseases (De Roos and Katan 2000; Marteau et al. 2001; Teitelbaum and Walker 2002).

Another way to influence the microbiota in the GIT is the intake of prebiotics. A prebiotic is defined as 'a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that have potential to improve host health' (Gibson and Roberfroid 1995). To be an effective prebiotic an ingredient must (i) neither be hydrolyzed nor absorbed in the upper part of the GIT; (ii) modulate the microbiota in the colon by stimulation of beneficial bacteria and inhibition of undesirable bacteria (Gibson and Fuller 2000; Gibson et al. 2000; Holzapfel and Schillinger 2002). Currently only carbohydrates act as prebiotic, in particularly non-digestible oligosaccharides (NDOs; Rastall and Gibson 2002). It appears that the effects of NDOs are predominantly the result of an increase in the proportion of bifidobacteria in the colon (Marks 2004; Ziemer and Gibson 1998). Bifidobacteria are able to metabolize the NDOs to organic acids and/or short-chain fatty acids (SCFAs), mainly lactic and acetic acid, which may be further metabolized to provide energy for the host (Manning and Gibson 2004). Most prebiotics are of relatively small size (degree of polymerization). It is assumed that the longer the oligosaccharides, the slower the fermentation, and the further the prebiotic effect will penetrate the colon (Manning and Gibson 2004; Voragen 1998). In Table 2 an overview is shown of potential prebiotics (Gibson and Angus 2000; Gibson et al. 2000; Hartemink 1999; Modler 1994). Fructo-oligosaccharides, inulin, lactulose, and transgalacto-oligosaccharides (β-linkage) have already proven their efficacy (Roberfroid 2002; Tuohy et al. 2003). Teitelbaum and Walker (2002), and Van Loo et al. (1999) reviewed the effects in human nutrition studies.

Poly- and/or oligosaccharides used as prebiotic can be produced using a range of different methods like extraction, chemical or enzymatic hydrolysis or synthesis (Gibson et al. 2000; Van Laere 2000; Voragen 1998). Examples of extraction are soybean oligosaccharides (raffinose and stachyose) from soy flour and inulin from chicory. For the production of lactulose a chemical approach is used (Schumann 2000). Enzymatic hydrolysis processes can be used for the production of oligosaccharides from plant polysaccharides (Voragen 1998). Another way to prepare oligosaccharides is the use of glycosyl transferases and glycosyl hydrolases. These enzymes can be used for the synthesis of oligosaccharides like transgalacto-oligosaccharides or fructo-oligosaccharides, which can act as prebiotic (Gibson et al. 2000; Van Laere 2000).

Synbiotic or eubiotic is a combination of probiotic and prebiotic and is defined as 'a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the GIT, by selectively stimulating the growth

and/or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare' (Gibson and Roberfroid 1995). To date, there have been a limited number of in vivo studies with humans on this subject (Bouhnik et al. 1996; Kießling et al. 2002; Schaafsma et al.1998).

TABLE 2: Non-digestible oligosaccharides as prebiotics[a]

Name	Source
Fructo-oligosaccharides	Oligofructose is produced from (i) partial enzymic hydrolysis from chicory inulin or (ii) synthesis from sucrose using the transglycosylation activity of a β-fructofuranosidase.
Gluco-oligosaccharides	Gentio-oligosaccharides are produced from glucose molecules transferred from a sucrose donor to a maltose acceptor by a glycosyl-transferase.
Inulin	A polydisperse β-(2-1) fructan from e.g. chicory, artichoke, garlic.
Isomalto-oligosaccharides	Manufactured from starch by enzymatic hydrolysis and using the transglycosylation activity of an α-glucosidase.
Lactosucrose	Non-reducing oligosaccharide produced from a mixture of lactose and sucrose using the transglycosylation activity of a β-fructofuranosidase.
Lactulose	A semi-synthetic disaccharide prepared from lactose by alkaline isomerisation.
Soybean-oligosaccharides	α-Galactosyl sucrose derivatives like raffinose and stachyose.
Transgalacto-oligosaccharides	Derived from lactose by transglycosylation reaction of β-galactosidase.
Xylo-oligosaccharides	Derived from xylan or arabinoxylan from e.g. corncobs, oats, and wheat by enzymatic hydrolysis.

[a] Gibson and Angus (2000), Gibson et al. (2000), Hartemink (1999), and Modler (1994)

POLY- AND OLIGOSACCHARIDE MODIFYING ENZYMES

Enzymes are single-chain or multiple-chain proteins that act as biological catalysts with the ability to promote specific chemical reactions under the mild conditions prevailing in most living organisms (Wilson 2000). Based on the nature of chemical reactions catalyzed, enzymes can be classified into one of six classes and each enzyme can be assigned to a unique four-figure code (EC number; IUB 1984). A whole range of enzymes like transferases, hydrolases, lyases, and isomerases are able to modify poly- and oligosaccharides. For microorganisms in the gut the most important enzyme group to degrade poly- and oligosaccharides to fermentable sugars are hydrolases.

Glycosyl hydrolases starting with EC number 3.2.1.x are able to cleave glycosidic bonds in the presence of water. The enzymatic hydrolysis of a glycosidic linkage generally involves acid catalysis. Two critical residues are needed in the catalytic centre: one should act as proton donor whereas the other acts as a nucleophile/base. In most cases these residues are aspartate and/or glutamate in glycosyl hydrolases. The hydrolysis can be separated into two distinct mechanistic classes: (i) those hydrolyzing the glycosidic linkages with net inversion of anomeric configuration (inverting mechanism), and (ii) those showing net retention (retaining mechanism) (Davies and Henrissat 1995; Koshland 1953; Sinnot 1990). Retaining glycosyl hydrolases have a double displacement mechanism in which a covalent glycosyl-enzyme intermediate is formed during the catalysis. The inverting enzymes have a single displacement mechanism (Sinnot 1990).

Retaining glycosyl hydrolases can be used for the synthesis of glycosidic linkages instead of hydrolysis. This reaction is called transglycosylation. A schematic overview of a hydrolysis and transglycosylation reaction is shown in Figure 1. Elongated and/or more branched oligosaccharides may exert a more prebiotic effect in the more distal colonic region due to a slower fermentation (Voragen 1998). This could be advantageous because main disorder of the gut takes place in the more distal areas of the colon (Gibson et al. 2000). Also multiple branching requires more and different enzymes for enzymatic hydrolysis before its complete fermentation (Fooks and Gibson 2002). Another advantage of a different degree of polymerization and/or branching might be the formation of less flatulent prebiotics (Cummings et al. 2001) and/or a lower osmolarity.

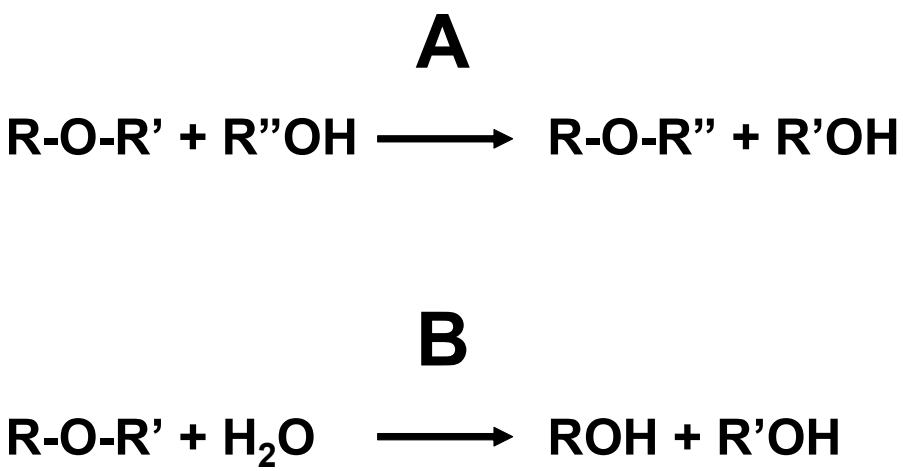

Figure 1: Schematic overview of a transglycosylation reaction (A) and a hydrolysis reaction (B) of glycosyl hydrolases. R, R', and R'' represents a sugar moiety.

BIFIDOBACTERIA

Bifidobacteria, as mentioned before, are considered to be one of the most important microorganisms in terms of human health. They rapidly colonize the intestinal tract of newly born infants and are the predominant species in the colon. Studies have shown that the number gradually decreases with age and also the composition of bifidobacteria will change (Hopkins et al. 2004; Mitsuoka 1990). Examination of the bifidobacteria populations has shown that there can be marked variation in the complexity and stability of *Bifidobacterium* populations between human subjects (Tannock 1998). Tissier (1900) has reported the first isolation of bifidobacteria in 1900. The microorganism was obtained from the feces of a child and it was named *Bacillus bifidus communis*. Nowadays, about 34 species (some different biotypes and subspecies) have been described (Table 3). The major habitat is considered to be the intestine of man and animals and about twelve species have been associated

with humans as host. Bifidobacteria are gram-positive, anaerobic, non-spore forming, non–motile, and catalase-negative bacteria (Biavati et al. 2000), and their rods are often Y- or V-shaped (Scardovi 1986). They are classified into the *Actinobacteria* family and contain a high G+C content (between 55-67%). Bifidobacteria are saccharolytic organisms that convert monosaccharides to acetic and lactic acid using the hexose fermentation pathway, also called fructose-6-phosphate shunt or bifid shunt, which is typical for bifidobacteria (De Vries 1969; Scardovi and Trovatelli 1965). Acetic and lactic acids are produced primarily in the molar ratio 3:2 (Biavati et al. 2000). Recently the genome of *B*. *longum* NCC2705 was sequenced and this revealed that more than 8.5% of the annotated genes were involved in carbohydrate metabolism (Schell et al. 2002), also preliminary results of the genome sequence of *B*. *breve* UCC2003 showed that more than 8% of the annotated genes are involved in sugar transport/metabolism (Van Sinderen 2004). This indicates that bifidobacteria are very well adapted in the colon and are able to utilize a whole range of (poly-) and oligosaccharides.

One of the main species of bifidobacteria in the colon of human adults is *B*. *adolescentis* (Matsuki et al. 1999). Reuter (1963) identified *B*. *adolescentis* as the first *Bifidobacterium* species, which predominate along with *B*. *longum* in the feces of human adults. It also occurs most frequently in sewage (Scardovi et al. 1979).

A multi-disciplinary research program was carried out at Wageningen Agricultural University in between 1995-1999 on the role of NDOs in human and animal nutrition (Alles 1998; Hartemink 1999; Houdijk 1998; Van Laere 2000). Van Laere (2000) studied the degradation of NDOs by intestinal bacteria and focused especially on the glycosyl hydrolases from *B*. *adolescentis*. She demonstrated that *B*. *adolescentis* produced glycosyl hydrolases, which are important for the carbohydrate fermentation in the colon. Different novel glycosyl hydrolases were identified and characterized. Some of the glycosyl hydrolases were able to show transglycosylation activity to produce elongated oligosaccharides. Interestingly, the cloning and expression of glycosyl hydrolases from *B*. *adolescentis* can lead to a higher production of these enzymes for further biochemical characterization and application for synthesizing potential prebiotics.

TABLE 3: Bifidobacteria and their habitat

Species	Habitat	Reference
B. adolescentis	Human adult	Reuter (1963)
B. angulatum	Human adult feces; sewage	Scardovi and Crociani (1974)
B. animalis		Masco et al. (2004) (Cai et al. (2000))
subsp. *animalis*	Rat feces	Scardovi and Trovatelli (1974) (Mitsuoka (1969))
subsp. *lactis*	Fermented milk; human, infant, rabbit, chicken feces; sewage	Meile et al. (1997)
B. asteroids	*Apis melifera*	Scardovi and Trovatelli (1969)
B. bifidum	Human adult, calf	Orla-Jensen (1924) (Tissier (1900))
B. boum	Piglet feces; bovine rumen; sewage	Scardovi et al. (1979)
B. breve	Calf, human	Reuter (1963)
B. catenulatum	Human adult (feces and vagina); sewage	Scardovi and Crociani (1974)
B. choerinum	Piglet feces	Scardovi et al. (1979)
B. coryneforme	Bee (*A. melifera*) intestine	Biavati et al. (1982)
B. cuniculi	Rabbit feces	Scardovi et al. (1979)
B. dentium	Human adult (oral cavity, feces, and vagina)	Scardovi and Crociani (1974)
B. gallicum	Human feces	Lauer (1990)
B. gallinarum	Chicken ceca	Watabe et al. (1983)
B. indicum	*A. cerana*, *A. dorsata*	Scardovi and Trovatelli (1969)
B. longum		Sakata et al. (2002)
biotype *infantis*	Infant, calf	Reuter (1963)
biotype *longum*	Human adult, infant, calf	Reuter (1963)
biotype *suis*	Pig feces	Matteuzzi et al. (1971)
B. magnum	Rabbit feces	Scardovi and Zani (1974)
B. merycicum	Bovine rumen	Biavati and Mattarelli (1991)

B. minimum	Sewage	Biavati et al. (1982) (Scardovi and Trovatelli (1974))
B. pseudocatenulatum	Sewage; breast and bottle-fed infant feces; calf feces; human adult feces	Scardovi et al. (1979)
B. pseudolongum		Yaeshima et al. (1992)
subsp. *globosum*	Bovine, cattle and sheep rumen; sewage; calf, cattle, chicken, dog, infant, lamb, pig, rabbit, and rat feces	(Biavati et al. (1982))
subsp. *pseudolongum*	Bull, calf, chicken, guinea pig, pig, rat	(Mitsuoka (1969))
B. psychraerophilum	Pig caecum	Simpson et al. (2004)
B. pullorum	Chicken feces	Trovatelli et al. (1974)
B. ruminantium	Bovine rumen	Biavati and Mattarelli (1991)
B. saeculare	Rabbit feces	Biavati et al. (1991)
B. scardovii	Human blood, urine, and hip	Hoyles et al. (2002)
B. subtile	Sewage	Biavati et al. (1982) (Scardovi and Trovatelli (1974))
B. thermacidophilum		Dong et al. (2000)
subsp. *thermacidophilum*	Anaerobic digester from bean-curd	
subsp. *porcinum*	Piglet feces	Zhu et al. (2003)
B. thermophilum	Calf, chicken, pig	Mitsuoka (1969)

AIM AND OUTLINE OF THE THESIS

The aim of the research described in this thesis was first to analyze the carbohydrate modifying enzyme toolbox of *B. adolescentis*: identification of the genes coding for such enzymes, their expression, and characterization to obtain more information about the potential of this microorganism to degrade poly- and oligosaccharides. The second objective was to study the

possibilities to synthesize tailor-made oligosaccharides using the glycosyl hydrolases from *B*. *adolescentis*. In this way oligosaccharides might be produced which are preferred prebiotic substrates for *B*. *adolescentis*.

Chapter 2 describes the construction of a genomic library of *B*. *adolescentis* in *Escherichia coli*. Screening of the library resulted in the identification of an α-galactosidase. The enzyme showed transglycosylation activity, and could be used for the production of α-galacto-oligosaccharides.

The cloning and characterization of two α-glucosidases is described in chapter 3. The two enzymes showed different substrate specificity and physico-chemical properties. Both enzymes showed different transglucosylation activities.

The cloning and characterization of a sucrose phosphorylase are described in chapter 4 and 5. In chapter 4 the physico-chemical properties are presented and the identification of a novel dimer produced by the sucrose phosphorylase. Chapter 5 deals with the 3D structure of this enzyme.

Chapter 6 reports the identification of genes encoding enzymes involved in the degradation of arabinoxylan. The biochemical characterization of the heterologously expressed arabinoxylan arabinofuranohydrolase-D3 is presented. The role of arabinoxylan oligosaccharide degrading glycosyl hydrolases of bifidobacteria in the colon is discussed.

Chapter 7 reviews bifidobacteria carbohydrases and prebiotics. In chapter 8 the role of *B*. *adolescentis* glycosyl hydrolases, characterized in this study, and their relation to prebiotics is discussed in more detail.

Finally, in chapter 9, the main conclusions and the concluding remarks of this study are presented.

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Chapter 2

Synthesis of α-galacto-oligosaccharides by a cloned α-galactosidase from *Bifidobacterium adolescentis*

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ABSTRACT

A genomic library of *Bifidobacterium adolescentis* was constructed in *Escherichia coli* and a gene encoding an α-galactosidase was isolated. The identified open reading frame showed high similarity and identity with bacterial α-galactosidases, which belong to family 36 of the glycoside hydrolases. For the purification of the enzyme from the medium a single chromatography step was sufficient. The yield of the recombinant enzyme was 100 times higher than from *B. adolescentis* itself. In addition to hydrolytic activity the α-galactosidase showed transglycosylation activity and can be used for the production of α-galacto-oligosaccharides.

INTRODUCTION

There is an increasing market for health-promoting microbial food supplements (probiotics) and health-promoting non-digestible food ingredients (prebiotics). Probiotics and prebiotics are claimed to improve gut health and thereby general well being. Non-digestible oligosaccharides belong to the group of prebiotics and are not digested in the upper part of the gastrointestinal tract. Examples are fructo-oligosaccharides, transgalacto-oligosaccharides, α-galacto-oligosaccharides, and oligosaccharides derived from plant cell wall polysaccharides by enzymatic hydrolysis (Van Laere et al. 1997; Voragen 1998).

Bifidobacteria produce enzymes, which are able to hydrolyze non-digestible oligosaccharides. *Bifidobacterium adolescentis* produces e.g. α- and β-galactosidase, β-xylosidase, and α-glucosidase (Van Laere et al. 1997). Besides hydrolase activity also some of the enzymes from bifidobacteria show transferase activity (Hung and Lee 1998; Nunoura et al. 1996; Van Laere et al. 1997; 1999). This transglycosylation activity of glycosidases can be used for the enzymatic synthesis of various oligosaccharides. These oligosaccharides might act as bifidobacteria growth promoting factors.

Recently, Leder et al. (1999) purified an α-galactosidase (EC 3.2.1.22) from *B. adolescentis* DSM 20083. Van Laere et al. (1997; 1999) reported that this enzyme had also transglycosylation activity. However, for industrial production of α-galacto-oligosaccharides larger amounts of α-galactosidase are needed. Therefore, the objective of this study was a high level production of α-galactosidase in a heterologous host. In this paper the cloning and expression of the α-galactosidase gene from *B. adolescentis* in *Escherichia coli* and the purification and characterisation of the heterologously expressed enzyme are described.

MATERIALS AND METHODS

Bacteria and culture conditions. *B. adolescentis* DSM 20083 was grown anaerobically at 37 ^{o}C in MRS medium pH 6.0 (Difco) containing 0.5 g cysteine l^{-1}. *Escherichia coli* XL1-Blue MRF′ (Promega) was used for cloning purposes and was grown in Luria-Bertani (LB) broth or on solidified (15 g agar l^{-1}) LB medium. Ampicillin was added to give 50 μg ml^{-1} when appropriate (Sambrook et al. 1989).

Construction of genomic library and screening. The genomic DNA of *B. adolescentis* was isolated using a modified Marmur procedure as described by Johnson (1994). Twenty ml of cell suspension adjusted to pH 7.5 were incubated with 50 mg of lysozyme and 1 mg of mutanolysin. The isolated chromosomal DNA was partially restricted with *Tsp*509 I (Life Sciences). Fragments with a size of 2–3 kb were ligated in a Lambda ZAP II pre-digested *Eco*RI/CIAP-treated vector (Stratagene) according to the instructions of the supplier. Instead of SOLR cells, XL1-Blue MRF´ cells were used in the mass excision protocol. Phage DNA was packaged with Packagene Lambda DNA Packaging System (Promega).

The genomic library was spread on solid LB/agar plates supplemented with ampicillin and 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). After 16 h at 37 ^{o}C the colonies were covered with a layer containing 7 g agarose l^{-1} in LB medium. 4-Methyl-umbelliferyl-α-D-galactoside (Sigma), 0.1 ml, 1 mM, was spread over the top agarose. The plates were incubated at room temperature for 10 minutes and fluorescence detected with an UV light. Two other substrates, 4-methyl-umbelliferyl-α-L-arabinoside and 4-methyl-umbelliferyl-β-D-xyloside, were also tested.

Isolation and characterization of recombinant α-galactosidase. *E. coli* XL1-Blue MRF´, which carried the gene for α-galactosidase, was grown for 24 h in M9 medium supplemented with 1 mg thiamine ml^{-1}, 1 mM IPTG, and 50 μg ampicillin ml^{-1} (Sambrook et al. 1989). Culture medium was centrifuged for 10 min at 7,500 *g* at 4 ^{o}C and the supernatant was used for the purification of α-galactosidase. The supernatant was diluted with water (1:1) and applied onto a Q-Sepharose (Pharmacia) anion exchange column at a flow rate of 113 cm h^{-1} at 4 ^{o}C. Elution took place with 20 mM sodium phosphate buffer (pH 7.0) and a linear gradient of 0–0.5 M NaCl. Fractions with the highest α-galactosidase activity were pooled and concentrated by ultra filtration using a 10K Omega membrane (Filtron). NaN_3 was added at 0.1 g l^{-1}.

SDS-PAGE gel electrophoresis was carried out on the Pharmacia PhastSystem, using PhastGel 10–15% gradient gels (Pharmacia) according to the instructions of the supplier. Coomassie brilliant blue staining was used for detection of proteins. Native molecular mass was estimated by gel permeation on a Superdex S-200 column. The column was calibrated with thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), and ribonuclease A (13.7 kDa). Elution was performed with 0.15 M NaCl in 20 mM sodium phosphate buffer (pH 6.0) at a flow rate of 30 cm h^{-1} with the aid of the SmartSystem (Pharmacia).

Protein concentration was determined according to Sedmak and Grossberg (1977) using bovine serum albumin as the standard.

Enzyme assays. The rate of hydrolysis was determined in 20 mM sodium phosphate buffer (pH 6.5) containing 0.2 g *p*-nitrophenyl-α-D-galactopyranoside l^{-1} (Sigma) at 37 °C. The reaction was stopped by adding an equal volume of 0.5 M glycine/NaOH buffer (pH 9) containing 2 mM EDTA. The colour formation was measured at 400 nm (molar extinction coefficient 13,700 M^{-1} cm^{-1}). For estimating the pH-optimum a range was used of pH 3.0–8.0 in McIlvain buffer (0.1 M citric acid/0.2 M *di*-sodium hydrogen phosphate) at 30 °C. The temperature-optimum was measured at 20–60 °C in 20 mM sodium phosphate buffer (pH 6.5). One unit of enzyme activity (U) is defined as the amount of enzyme that liberates 1 μmol *p*-nitrophenol min^{-1} under the specified conditions.

Transferase activity was measured with 0.04 U of α-galactosidase and 0.3 M melibiose or stachyose in McIlvain buffer (pH 8.0) for 4 h at 40 °C. Reaction products were analysed with High Performance Anion Exchange Chromatography (HPAEC). A Dionex CarboPac PA-100 (4 x 25 mm) and a Dionex pulsed electrochemical detection detector in the pulsed amperometric detection mode were used (Dionex). A gradient of 0 to 200 mM sodium acetate in 0.1 M NaOH was used from 0 to 30 min.

DNA sequencing and sequence analysis. The nucleotides of the α-galactosidase encoding gene were determined using an automated DNA Sequencer 373 (Applied Biosystems Inc.). The nucleotide sequence has been submitted to the GenBank Nucleotide Databases under the accession number AF124596.

RESULTS AND DISCUSSION

Construction and screening of a *B. adolescentis* genomic library. The yield of the primary *B. adolescentis* genomic Lambda ZAP II library was 8.0×10^4 PFU ml^{-1}. Restriction analysis of the excised plasmids showed that 94% of the colonies had an insert. The average size of the ligated fragments was 2.5 kb. This resulted in a genomic library from *B. adolescentis* of 93 Mb. The genome size of some *Bifidobacterium* spp. is in the range of 1.1–2.2 Mb as estimated by pulsed-field gel electrophoresis (Roy et al. 1996). This library represents approximately 40–45 times the genome of *B. adolescentis*.

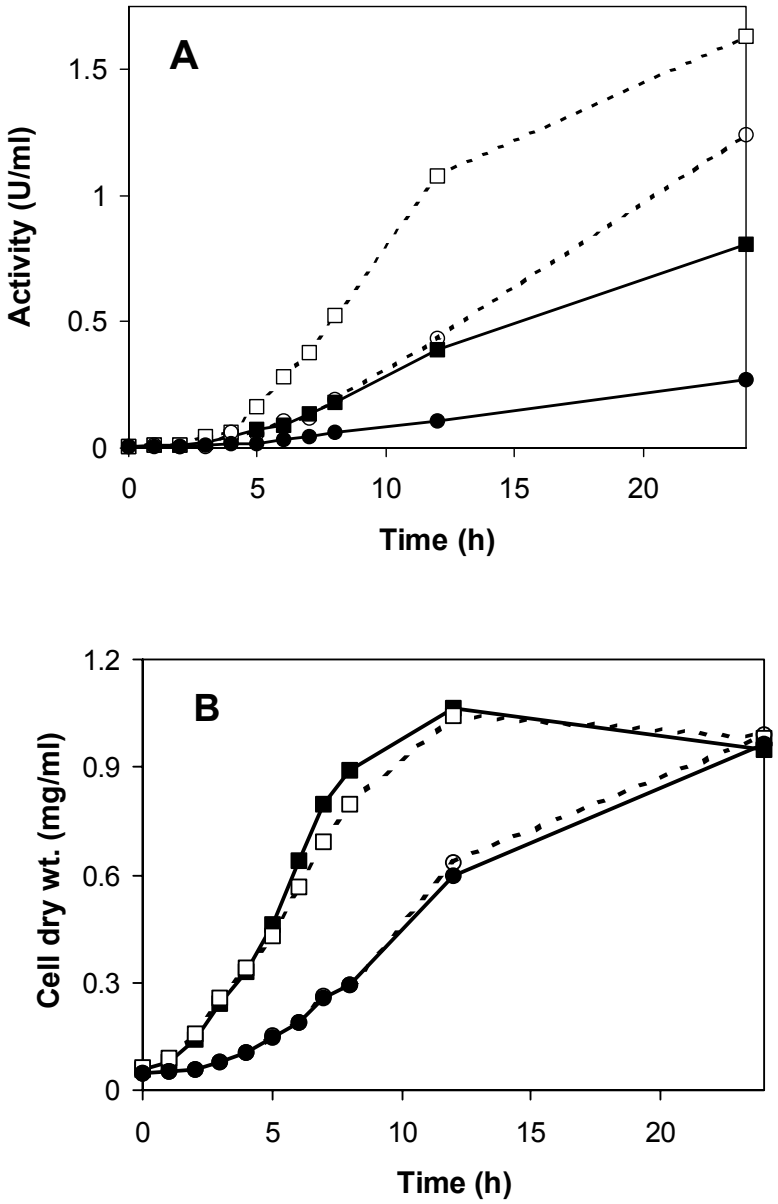

Figure 1: α-Galactosidase production into the media (**A**) and growth curve (**B**) of *Escherichia coli*, which contains the α-galactosidase gene *pAGA* from *Bifidobacterium adolescentis*. *E. coli* was first grown overnight in LB medium and sequentially diluted and grown in the different media. The results are the means of two independent experiments. *Filled squares* LB medium, *open squares* LB medium with IPTG, *filled circles* M9 medium, and *open circles* M9 medium with IPTG. The enzyme activity is explained in materials en methods.

For screening of α-galactosidase activity producing *E. coli* transformants 40,000 colonies were plated and tested. Five colonies gave a fluorescent halo when incubated with 4-methyl-umbelliferyl-α-D-galactoside. No fluorescent signal was detected with 4-methyl-umbelliferyl-α-L-arabinoside or 4-methyl-umbelliferyl-β-D-xyloside as substrate. Plasmid of each of the positive clones was isolated and incubated with the restriction enzymes *Xho*I and *Eco*RI. A similar restriction pattern was found for all five clones. One of the plasmids, designated *pAGA,* was used for further analysis.

Sequence analysis. The complete sequence of the 2.85 kb insert was determined. An ORF of 2,403 nucleotides was detected on this fragment (GenBank accession number AF124596). A putative ribosome-binding site was found 6 bp upstream of an initiation codon (at 109 bp). The deduced amino acid sequence contains 764 amino acid residues, encoding a protein of 84,242 Da.

Most of the α-galactosidases belongs to family 27 or 36 of the glycoside hydrolases. A strong relationship is reported between these two families, which resulted in the clan group GH-D (Henrissat and Bairoch 1996). The deduced amino acid sequence showed high similarity and identity with published α-galactosidase from *E. coli* (Aslanidis et al. 1989), *Streptococcus mutans* (Aduse-Opoku et al. 1991), and *Thermotoga neapolitana* (King et al. 1998) of family 36. Less similarity and identity was found with α-galactosidases of family 27 (Den Herder et al. 1992; Overbeeke et al. 1989; Shibuya et al. 1998; Zhu and Goldstein 1994). Therefore, we concluded that the α-galactosidase from *B. adolescentis* belongs to family 36.

Expression and characterization of the recombinant α-galactosidase. The production of α-galactosidase and the growth curve of *E. coli* containing the α-galactosidase gene *pAGA*, in two different media is shown in Figure 1. A 2–5 fold increase in enzyme activity was observed when IPTG was added to the media (Figure 1A). Addition of IPTG had no effect on the cell growth (Figure 1B). This indicated that the gene was under the control of the *lac* promoter of *p*Bluescript phagemid.

Anion exchange chromatography was used to purify the recombinant α-galactosidase. The enzyme appeared to be pure, as judged with SDS-PAGE electrophoresis and Coomassie brilliant blue staining. The starting material contained 108 U of α-galactosidase activity, whereas the purified enzyme after concentration contained a total of 83 U. The purification factor was 5.2. The physico-chemical properties of the recombinant enzyme were determined and are shown in Table 1. In *B. adolescentis* only the presence of one α-galactosidase was detected (Leder et al. 1999; Van Laere et al. 1997; 1999). Leder et al. (1999) and Van Laere et al. (1999) found a molecular mass of

79 and 83 kDa, respectively, using SDS-PAGE electrophoresis. The deduced molecular mass of the recombinant α-galactosidase of 84 kDa corresponds with these values. Both suggested that the native α-galactosidase consist of four subunits in *B*. *adolescentis*. Also in *E*. *coli* the recombinant α-galactosidase consists of four subunits as judged with gel permeation chromatography. Based on these results it can be concluded that the cloned α-galactosidase is the same as described by Leder et al. (1999) and Van Laere et al. (1999).

Table 1: Physico-chemical properties of the recombinant α-galactosidase from *Bifidobacterium adolescentis*

Specific activity towards *p*-nitrophenyl-α-D-galactopyranoside (U/mg)	388
Native molecular mass (kDa)	332
Molecular mass (kDa; SDS-PAGE)	83
Optimum pH	6.5
Optimum temperature (^{o}C)	45

However, the yield of the α-galactosidase from *B*. *adolescentis* in *E*. *coli* is higher than in *B*. *adolescentis* itself. From a cell-free extract from 1 litre *B*. *adolescentis* fermentation medium Leder et al. (1999) purified 0.035 mg protein and the purification scheme contained several steps. In our study 3.56 mg protein was purified from 1 l M9 medium when 1 mM IPTG was added. Approximately 100 times more α-galactosidase is obtained with the recombinant enzyme. In addition, the recombinant α-galactosidase was obtained pure after one single chromatography step.

Besides hydrolytic activity, the α-galactosidase from *B*. *adolescentis* also showed transglycosylation activity (Van Laere et al. 1997; 1999). In our study melibiose and stachyose were used as substrate for the synthesis of higher oligomers by the cloned α-galactosidase (Figure 2). Besides transglycosylation some hydrolysis occurred. In the case of melibiose the monomers galactose and glucose were produced (peak AI), whereas galactose (peak BI) was released using stachyose as substrate. Both substrates could be used for the production of α-galacto-oligosaccharides (peak AIV and BIV). These oligosaccharides will be characterised and tested for their potential as bifidobacteria growth promoting factors. The recombinant α-galactosidase will be used for the higher-level production of α-galacto-oligosaccharides.

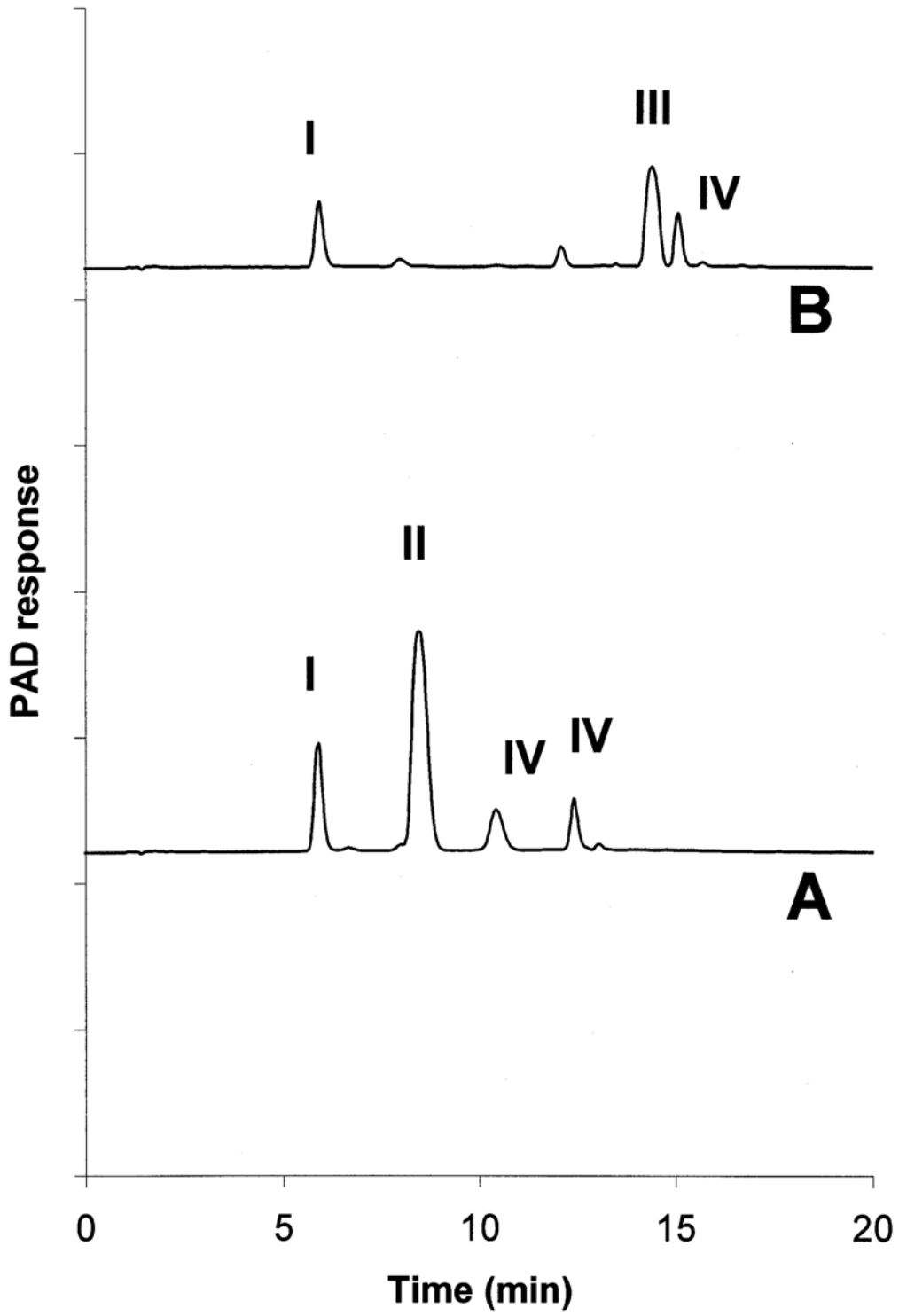

Figure 2: HPAEC elution pattern of the reaction products formed after transglycosylation of melibiose (**A**) or stachyose (**B**) by the recombinant α-galactosidase from *Bifidobacterium adolescentis*. Galactose and glucose (AI), galactose (BI), melibiose (II), stachyose and raffinose (III), and α-galacto-oligosaccharides (IV). Pulsed amperometric detection (PAD).

Another subject of further investigation is to produce enough enzyme for crystallography studies to elucidate the three dimensional structure of the enzyme. At this moment the three-dimensional structure of family 36 of the glycoside hydrolases is not known yet (Henrissat and Bairoch 1996). The high production of the recombinant α-galactosidase makes this possible.

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Chapter 3

Cloning and characterization of two α-glucosidases from *Bifidobacterium adolescentis* DSM20083

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Applied Microbiology and Biotechnology (2003) 61: 55–60

ABSTRACT

Two α-glucosidase encoding genes (*aglA* and *aglB*) from *Bifidobacterium adolescentis* DSM20083 were isolated and characterized. Both α-glucosidases belong to family 13 of the glycoside hydrolases. Recombinant AglA (EC 3.2.1.10) and AglB (EC 3.2.1.20), expressed in *Escherichia coli*, showed high hydrolytic activity towards isomaltose and *p*np-α-glucoside. The K_m for *p*np-α-glucoside was 1.05 and 0.47 mM and the V_{max} was 228 and 113 U mg^{-1} for AglA and AglB, respectively. Using *p*np-α-glucoside as substrate, the pH optimum for AglA was 6.6 and the temperature optimum was 37 ^{o}C. For AglB, values of pH 6.8 and 47 ^{o}C were found. AglA also showed high hydrolytic activity towards isomaltotriose and, to a lesser extent, towards trehalose. AglB has a high preference for maltose and less activity towards sucrose; minor activity was observed towards melizitose, low molecular weight dextrins, maltitol, and maltotriose. The recombinant α-glucosidases were tested for their transglucosylation activity. AglA was able to synthesize oligosaccharides from trehalose and sucrose. AglB formed oligosaccharides from sucrose, maltose, and melizitose.

INTRODUCTION

In the food industry there is increasing interest to positively influencing the intestinal microflora through the diet by probiotics and/or prebiotics. A probiotic can be defined as 'a preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effect in this host' (Schrezenmeir and De Vrese 2001). Prebiotic is 'a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that have the potential to improve host health' (Gibson and Roberfroid 1995). When probiotics and prebiotics are used in combination this is named synbiotics.

Bacteria used as probiotic are mainly from the genera *Lactobacillus* or *Bifidobacterium* and it is now widely accepted that an effective human probiotic should be of human origin (O'Sullivan 2001). Among bifidobacteria the species *Bifidobacterium adolescentis* forms (one of) the largest group of inhabitants of the large intestine in human adults (Matsuki et al. 1999). Most potential prebiotics are carbohydrates and it is often assumed that they should increase the number and/or activity of lactic acid bacteria and/or bifidobacteria.

Starches that are resistant to – or escape digestion by – pancreatic amylase in the small intestine are defined as resistant starch (Englyst et al. 1992). They can be degraded and fermented by human gut bacteria (Englyst and Macfarlane 1986). It is known that the interaction of resistant starch with the gut flora throughout the digestive tract can promote human health (Bird et al. 2000). However, resistant starch is classified not as a prebiotic but as a colonic food because different bacteria can ferment resistant starch (Gibson and Roberfroid 1995). On the other hand, more recent animal fed studies with resistant starch showed the highest enumeration of lactic acid bacteria and bifidobacteria (Kleessen et al. 1997; Silvi et al. 1999). This makes it interesting to study the enzyme activities that play a role in the degradation of starch and α-gluco-oligosaccharides. Therefore, the amylolytic and α-glucosidase activity of *B. adolescentis* were investigated by screening a genomic library (chapter 2; Van den Broek et al. 1999).

Here, we report the identification of two different α-glucosidase-encoding genes (*aglA* and *aglB*) from *B. adolescentis*. The α-glucosidases were identified by their ability to hydrolyze 4-methyl-umbelliferyl-α-D-glucoside.

MATERIALS AND METHODS

Microorganisms and growth conditions. *Escherichia coli* XL1-Blue MRF′ (Promega), used for initial cloning and plasmid isolation, was grown in Luria-Bertani (LB) broth or on LB agar plates at 37 ^{o}C. Ampicillin was added when appropriate, to a concentration of 50 mg l^{-1}. For purification of the recombinant α-glucosidases, the *E. coli* cells, carrying a plasmid-based *aglA* or *aglB* gene, were cultivated overnight in LB broth with ampicillin (LAMP). For production of AglA, the medium was supplemented with 1 mM iso-propyl *β*-D-thiogalactopyranoside (IPTG).

To study the effect of glucose and IPTG on the expression of *aglB*, *E. coli* cells carrying the *aglB* gene were grown overnight in LAMP medium. Volumes of 3-ml fresh LAMP medium were inoculated with these cells to an optical density of 0.1 at 600 nm (OD_{600}). The media were supplemented (or not) with 1 mM IPTG and/or 20 g glucose l^{-1}. Cells were grown for 7.5 hours at 37 ^{o}C (200 rpm) and after centrifugation for 10 min at 7,500 *g* the supernatant was tested for α-glucosidase activity.

To measure the total α-glucosidase activity in cell-free extract and cell culture supernatant, *E. coli* cells were grown overnight in 25 ml LAMP medium. In the case of *E. coli* cells carrying the *aglA* gene, the medium was supplemented with 1 mM IPTG. After growth, cells were centrifuged for 10 min at 3,660 *g* and the supernatant was collected. The pellet was re-suspended in 10 ml 20 mM potassium phosphate buffer (pH 6.5), and the cells were disrupted by sonic treatment (10 min; duty cycle 30%; Branson Sonifier 250) on ice. This step was repeated twice to obtain cell-free extract.

Genomic library and screening. The genomic library of *B. adolescentis* DSM20083 was constructed in a Lambda ZAP II vector (Stratagene) as described (chapter 2; Van den Broek et al. 1999). The screening for α-glucosidase activity was performed according to Van den Broek et al. (1999; chapter 2) using 4-methyl-umbelliferyl-α-D-glucoside (Sigma) as substrate. For α-amylase activity, the genomic library was spread on LB agar plates containing 0.25 g AZCL-amylose l^{-1} (Megazyme) and grown overnight at 37 ^{o}C. The LB agar plates were supplemented with 50 mg ampicillin l^{-1} and 1 mM IPTG.

Southern blot analysis was performed as described by Sambrook et al. (1989). Chromosomal DNA was digested with *Hinc*II (Fermentas) and hybridized with the coding part of *aglA* or *aglB*. The DIG non-radioactive nucleic acid labeling and detection system was used according to the instruction of the supplier (Roche).

Isolation and characterization of recombinant α-glucosidases. *E. coli* cell culture (1 l) containing the recombinant AglA was centrifuged for 10 min at 8,000 *g* at 4 °C, and the cells were washed once with 50 mM sodium acetate (pH 4.5). The cells were re-suspended in 50 ml of the same buffer and disrupted by sonic treatment (10 min; duty cycle 30%; Branson Sonifier 250) on ice. Subsequently, the suspension was centrifuged for 15 min at 20,000 *g* at 4 °C, the supernatant was collected and the pellet was re-suspended in 50 ml sodium acetate buffer (pH 4.5) and another sonic treatment was performed. This step was repeated three times. The cell-free extracts obtained were pooled and applied to a Q-Sepharose (Amersham) anion exchange column. Elution took place with a linear gradient of 0–0.5 M NaCl in 50 mM sodium acetate (pH 4.5) at a flow rate of 57 cm h^{-1}. Fractions with the highest α-glucosidase activity were pooled and further purified on a Superdex 200 PG (Amhersham) gel permeation column and eluted with 0.15 M NaCl in 20 mM potassium phosphate buffer (pH 6.5) at a flow rate of 30 cm h^{-1}.

Cell culture (1 l) containing AglB was centrifuged for 10 min at 8,000 *g* at 4 °C, and the supernatant was filtrated through a 0.2 μm filter. The filtrate was dialyzed overnight against 50 mM sodium acetate buffer (pH 4.5). The same columns and conditions as described for AglA were used for the purification of AglB.

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as the standard. SDS-PAGE was carried out on the Pharmacia PhastSystem according to the instructions of the supplier. Coomassie brilliant blue staining was used for detection of proteins on the PhastGel 10-15% gradient gels (Amersham). The native molecular mass was estimated by gel exclusion chromatography using the Äkta Purifier equipped with a Superdex 200 HR column (Amersham). The column was calibrated with thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa). Elution was performed with 20 mM potassium phosphate buffer (pH 6.5) containing 0.15 M NaCl at a flow rate of 76 cm h^{-1}.

N-Terminal amino acid sequence determination was carried out at the Sequence Centre of Utrecht University (The Netherlands) and was performed as described before (Van Casteren et al. 2000). For staining 10 g Coomassie brilliant blue l^{-1} in 40% (v/v) methanol and 5% (v/v) acetic acid was used. Destaining was in 10% (v/v) acetic acid in 50% (v/v) methanol.

Enzyme assays. Alpha-glucosidase activity was determined with 0.2 g *p*-nitrophenyl-α-D-glucopyranoside l^{-1} (Sigma) in 20 mM potassium phosphate buffer (pH 6.5) at 30 °C, essentially as described before (chapter 2; Van den Broek et al. 1999). One unit (U) of enzyme activity was

defined as the amount of enzyme that liberates 1 μmol *p*-nitrophenol min^{-1} under the specified conditions. The molar extinction coefficient of 13,700 M^{-1} cm^{-1} was used for calculation of the enzyme activity. This assay was also used to measure the temperature-optimum between 4 and 70 ^{o}C. For determination of the pH-optimum McIlvain buffers (0.1 M citric acid/0.2 M *di*-sodium hydrogen phosphate buffer) in the range pH 2.5–7.5 were used. For kinetic experiments, the substrate concentration in the standard assay was varied in the range of 0.025–10 mM.

The hydrolytic activity of AglA and AglB towards substrates with different glucosidic linkages was measured with high performance anion exchange chromatography (HPAEC). A Dionex CarboPac PA-100 (4 × 250 mm) column and a pulsed electrochemical detection detector in the pulsed amperometric detection mode were used (Dionex). A flow rate of 1 ml min^{-1} was used with the following gradient of sodium acetate in 0.1 M NaOH: 0–5 min, 0 mM; 5–20 min, 0–150 mM; 20–25 min, 150–1,000 mM; 25–30 min, 1,000 mM. For maltitol the following gradient was used : 0–26 min, 16 mM NaOH; 26–33 min, 16–100 mM NaOH; 33–38 min, 0–1,000 mM sodium acetate in 100 mM NaOH; 38–43 min, 1,000 mM sodium acetate in 100 mM NaOH. The incubations were performed with 2.5 g substrate l^{-1} in 100 mM potassium phosphate buffer (pH 6.5) and 8 mU ml^{-1} AglA or AglB for 22 h at 30 ^{o}C. The reactions were stopped by boiling the incubation mixtures for 10 minutes. Cellobiose was obtained from BDH, dextrins from Spreda, sucrose from Merck and all other substrates from Sigma.

Transglycosylation activities were performed with 0.3 M maltose, melizitose, trehalose, or sucrose in 100 mM potassium phosphate buffer (pH 6.5) and 80 mU ml^{-1} AglA or AglB at 30 ^{o}C for 16 h. The reaction was stopped by boiling for 10 minutes. After centrifugation for 10 min at 10,000 *g* the supernatant was analyzed by HPAEC using the same gradient as described above.

DNA sequencing and sequence analysis. An automated DNA Sequencer 373 (Applied Biosystems) was used to determine the nucleotides of the genes. The DNA sequence data have been submitted to the GenBank nucleotide databases under the accession number AF358444 (*aglA*) and AF411186 (*aglB* and *ccp*). The BLAST2 program (Altschul et al. 1997) was used for searching sequence homologies.

RESULTS

Identification of α-glucosidase and α-amylase genes from *B. adolescentis*. Clones from the genomic library were plated on agar plates for the detection of enzyme activities. Plasmids from *E. coli* transformants were isolated from colonies that gave a fluorescent halo when incubated with 4-methyl-umbelliferyl-α-D-glucoside. Two different types of plasmids were isolated with an insert of 2.0 kb and 3.7 kb, respectively. No transformants could be identified showing α-amylase activity with AZCL-amylose as substrate under the conditions used.

The complete nucleotide sequence of both inserts was determined. The smallest insert (2,043 nucleotides) contained the 3´-end of a partial open reading frame (ORF) of 1,815 bp (*aglA*; AF358444). The deduced amino acid sequence showed 52% identity with a putative α-glucosidase from *Brevibacterium fuscum* var. *dextranlyticum* (AB025195) and 49, 48, 49, and 48% identity with oligo-1,6-glucosidases from *Bacillus thermoglucosidasius* (P29094), *Bac. cereus* (P21332), *Bac. halodurans* (AP001517), and an α-amylase domain from *Bac. anthracis* (NP658052), respectively. These α-glucosidases are members of family 13 of the glycoside hydrolases (Henrissat 1991) and, therefore, AglA was classified as a putative α-glucosidase belonging to this family 13.

The largest insert (3,708 nucleotides; AF411186) contained two ORFs. The first consisted of 1,035 nucleotides and encoded a 344 amino acid polypeptide of 37,401 Da. The deduced amino acid sequence showed 30, 29, 28, 29, and 27% identity with transcriptional repressors or catabolite control proteins of *Streptomyces coelicolor* (AL583943), *Vibrio cholerae* (H82046), *Yersinia pestis* (AJ414141), *Themoanaerobacter tengcongensis* (AE13146), and *Bac. megaterium* (P46828), respectively. This putative transcriptional repressor (designated *ccp*) contains a DNA-binding helix-turn-helix motif that has been found in regulatory proteins of the LacI/GalR family (Weickert and Adhya 1992). The initiation codon of *ccp* was preceded with a putative ribosomal binding site.

The second ORF on the 3.7 kb insert consisted of 1,773 nucleotides. The gene, designated *aglB*, encodes a protein of 590 amino acids with a calculated molecular mass of 66,575 Da. *AglB* and the gene encoding Ccp are transcribed in opposite directions. A database search with the deduced amino acid sequence of *aglB* revealed 50, 44, 45, 45, and 45% identity with α-glucosidases from *S. coelicolor* A3(2) (AL442629), *S. coelicolor* A3(2) (AL355752), *Thermomonospora curvata* (AF105219), *S. coelicolor* A3(2) (AL353864), and *S. lividans* (T30268), respectively. All these α-glucosidases are members of family 13 of the glycoside hydrolases and, therefore, AglB was also classified into this family. The identity and similarity of *aglA* with *aglB* is lower, 31 and 41%,

respectively. According to Chiba (1997) α-glucosidases can be classified into two families based on recognition of homologous sites of amino acid sequences. The conserved blocks of amino acid sequences of AglA and AglB (Figure 1) are typical for family I, members of which are found in bacteria, insects, or yeast.

Deduced catalytic sites region	Region				
	1	2	3	4	5
AglA	114-***D***L***V***V***NH***	217-***G***F***R***M***D***VIT	290-***E***APG	361-FFC***NHD***	
AglB	108-***D***I***V***P***NH***	208-***G***F***R***V***D***VAG	274-***E***AWW	330-VMS***NHD***	
Family I	**D**X**V**X**NH**	**G**X**R**X**D**XXXX	**E**XXX	XXX**NHD**	
Family II		**G**X**W**X**DM**X**E**X			**G**X**D**X**CGF**

Figure 1: Alignment of the conserved amino acid sequences of AglA and AglB of *Bifidobacterium adolescentis* and the consensus of conserved amino acid sequences of family I and II of the α-glucosidases (Chiba 1997; Krasikov et al. 2001). The main conserved amino acids are in *bold* and the amino acids of AglA and AglB in common with family I are in *bold italics*. *X* any amino acid.

Southern blot analysis, of digested chromosomal DNA from *B. adolescentis* hybridized with the coding part of the *aglA* or *aglB* gene, confirmed the presence of both genes in *B. adolescentis*.

Purification and physico-chemical properties of AglA and AglB. Most of the total α-glucosidase activity of the recombinant AglA was found in the cell-free extract after induction with IPTG. The total activity of a 25 ml cell culture was 30 U in the cell-free extract and 5 U in the cell culture supernatant. The cell-free extract was used as starting material for the purification of AglA by anion exchange and size exclusion chromatography. The total α-glucosidase activity of a 25 ml cell culture of the transformant carrying the *aglB* gene was 115 U in the cell-free extract and 11 U in the cell culture supernatant. The cell culture supernatant contained a relatively higher total amount of α-glucosidase activity, as compared with AglA, and was used as starting material for purification. For the production of this recombinant α-glucosidase, the transformant was cultivated in M9

medium with IPTG as inducer and glucose as carbon source. However, under these conditions, low levels of α-glucosidase activity were obtained. To investigate the role of IPTG as inducer and glucose as carbon source, the transformant was grown in LB media with and without glucose and/or IPTG. The addition of glucose resulted in a much lower level of α-glucosidase activity. It was also noticed that the expression of the *aglB* was not induced by IPTG, because the same or even lower levels of activity were measured without addition of IPTG. The recombinant AglB was purified from the culture fluid of a culture grown in LB medium. The same chromatography steps were used for AglA and AglB. Both enzymes eluted at 150 mM NaCl using the anion exchange column. As judged with SDS-PAGE and Coomassie brilliant blue staining both enzymes appeared to be pure after size exclusion chromatography step. The physico-chemical properties of both enzymes are given in Table 1.

The *aglA* gene did not contain a starting codon. For expression of AglA the inducible *lac* promotor upstream from the *lacZ* gene of the *p*Bluescript vector can give a fusion protein with the N-terminal portion of the β-galactosidase. Attempts were made to determine the N-terminal amino acid sequence of the recombinant AglA. However, only very low signals were obtained, which indicates that the N-terminus of AglA was blocked. The truncated *aglA* encodes a polypeptide with a calculated molecular mass of 68,276 Da. The N-terminal portion of *lacZ* encodes for a protein of 3,862Da. The fusion protein was thus assumed to have a molecular mass of 72,138 Da.

TABLE 1: Physico-chemical properties of recombinant α-glucosidases from *Bifidobacterium adolescentis*

	AglA[a]	AglB
K_m (mM)[b]	1.05 ± 0.15	0.47 ± 0.03
V_{max} (U mg^{-1})[b]	228 ± 10	113 ± 2
Deduced molecular mass (Da)	72,138	66,575
Molecular mass (kDa; SDS-PAGE)	71	73
Native molecular mass (kDa)	68	149
Optimum pH	6.6	6.8
Optimum temperature (°C)	37	47

[a] Translational fusion of N-terminal portion of β-galactosidase (*LacZ*)
[b] *p*-Nitrophenyl-α-D-glucopyranoside as substrate; mean of four measurements ± S.E

TABLE 2: Substrate specificity of recombinant α-glucosidases from *Bifidobacterium adolescentis*[a]

Substrate[b]	AglA	AglB
*p*np-Glucopyranoside (α)	++++	++++
Trehalose (α-1,1)	++	–
Sucrose (α-glc-1,2 *p*βfru)	–	++
Melizitose (α-glc-1,3 *p*βfru)	–	+
Amylose (α-1,4)	–	–
Low molecular weight dextrin (α-1,4)	–	+
High molecular weight dextrin (α-1,4)	–	–
Maltitol (α-1,4)	–	+
Maltose (α-1,4)	–	+++
Maltotriose (α-1,4)	–	+
Maltotetraose (α-1,4)	–	–
Maltopentaose (α-1,4)	–	–
Nigeran (α-1,4/α-1,3)	–	–
Amylopectin (α-1,4/α-1,6)	–	–
Pullulan (α-1,4/α-1,6)	–	–
Cellobiose (β-1,4)	–	–
Isomaltose (α-1,6)	++++	+++
Isomaltotriose (α-1,6)	++++	–
Dextran (α-1,6/α-1,3/α-1,4)	–	–
Melibiose[c]	–	–

[a] Glucose release under the conditions used, as described in Materials en methods (scale: – 0–0.01 μmol glucose, + 0.01–0.05 μmol glucose, ++ 0.05–0.10 μmol glucose, +++ 0.1–1.0 μmol glucose, ++++ >1.0 μmol glucose)
[b] Type of linkage in brackets
[c] Glucose at the reducing end (Gal-α-1,6-Glc)

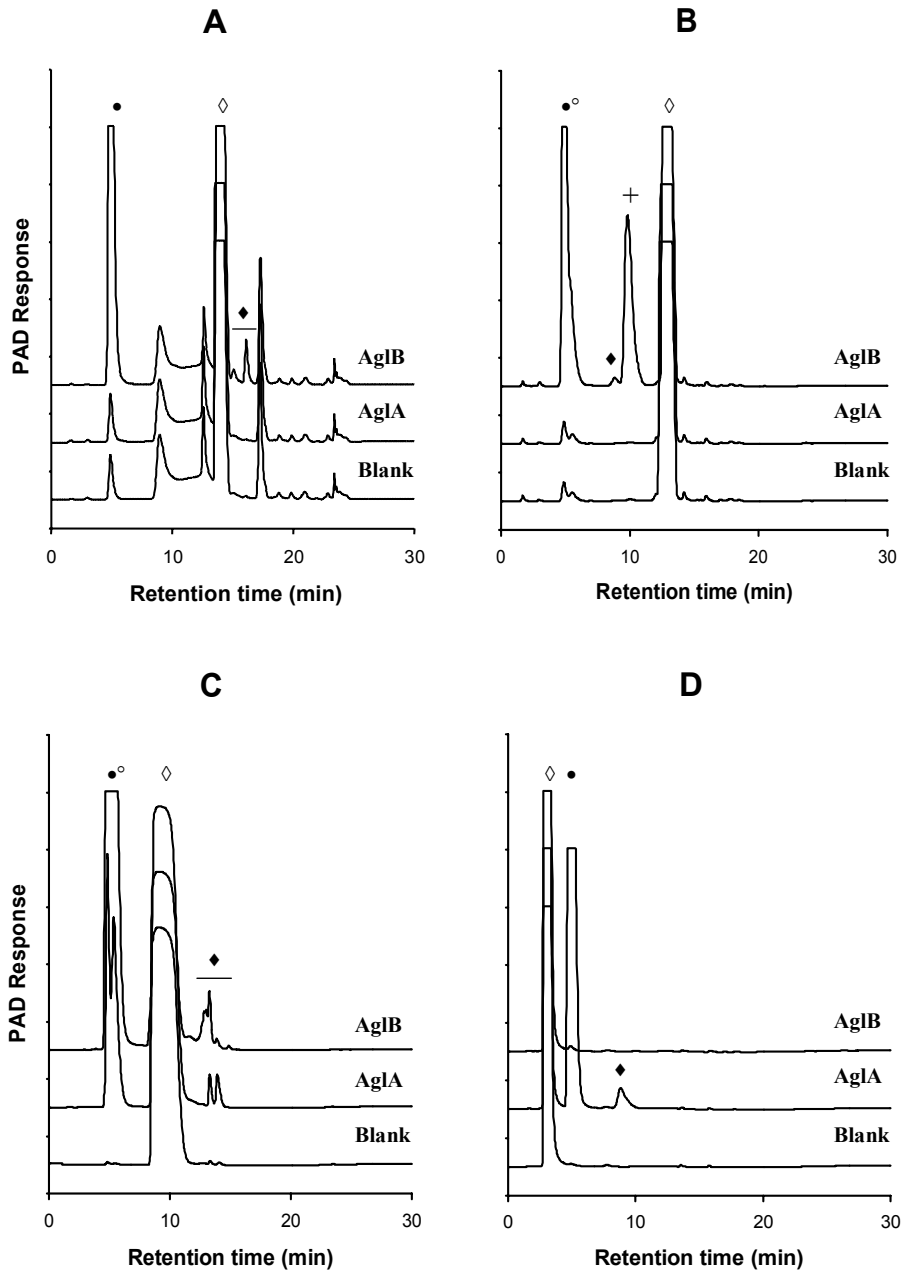

Figure 2: High performance anion exchange chromatography (HPAEC) of reaction products formed after transglucosylation of maltose (**A**), melizitose (**B**), sucrose (**C**), or trehalose (**D**) by the recombinant α-glucosidases AglA and AglB from *Bifidobacterium adolescentis*. *Filled circles* Glucose, *open circles* fructose, *open diamonds* substrate, + sucrose, *filled diamonds* newly synthesized α-glucosides (*underlined diamond* more than one main product). *PAD* pulsed amperometric detection (PAD).

Substrate specificity and transglycosylation reaction. In addition to their activity towards *p*np-α-glucoside, both enzymes were tested for their ability to degrade glucose-containing substrates. The reaction products were analyzed by HPAEC. AglA showed the highest activity against *p*np-α-glucoside, isomaltose, and isomaltotriose; somewhat lower activity was observed with trehalose (Table 2). AglB showed the highest activity towards *p*np-α-glucoside. Isomaltose and maltose were also degraded to a large extent. Lower activities were obtained with sucrose, maltitol, maltotriose, melizitose, and lower molecular weight dextrins. No detectable activity was noticed against isomaltotriose. In general α-glucosidases are classified as EC 3.2.1.20 and have a relative high hydrolytic activity towards maltose. Oligo-1,6-glucosidases have obtained EC number 3.2.1.10. AglA showed high activity against oligo-1,6-glucosides and has high amino acid sequence identities and similarities with other oligo-1,6 glucosidases. Therefore, AglA was classified as EC 3.2.1.10. AglB, on the other hand, shared most characteristics with α-glucosidases having EC 3.2.1.20.

The potential for catalysis of transglycosylation reactions was tested for both enzymes. Maltose, melizitose, sucrose, and trehalose were used as substrate. AglA was able to produce oligosaccharides from trehalose (Figure 2D). Two transglycosylation products were also synthesized from sucrose (Figure 2C), although no detectable hydrolysis activity was observed when sucrose was incubated with a lower amount of enzyme (Table 2). AglB synthesized oligosaccharides from maltose, sucrose, and melizitose (Figure 2). With sucrose as substrate the two enzymes produced a different ratio of oligosaccharides. For example, AglB produced an oligosaccharide that eluted at the same retention time as melizitose, while this peak was absent using AglA as enzyme. Incubation of AglB with maltose synthesized products, which eluted earlier than maltotriose. The main hydrolytic products obtained from melizitose by action of AglB were glucose and sucrose. A small amount of synthesized oligosaccharides eluted just before sucrose.

DISCUSSION

Two α-glucosidase genes (*aglA* and *aglB*) were identified by screening a genomic library of *B. adolescentis* DSM20083 with 4-methyl-umbelliferyl-α-D-glucoside as substrate. Alpha-glucosidases are exoenzymes that hydrolyze terminal glycosidic bonds, releasing α-glucose from the non-reducing end of the substrate chain (Krasikov et al. 2001). Several studies dealing with bifidobacteria have reported the presence of α-glucosidase activity (Chevalier et al. 1990;

Desjardins et al. 1990; O'Brien and Mitsuoka 1991; Rada 1997; Tochikura et al. 1986; Van Laere et al. 2000; Wang et al. 1999). However, purification and partial characterization of their α-glucosidases has been described only for *B*. *adolescentis* (Igaue et al. 1983a; 1983b) and *B*. *pseudolongum* (Degnan and Macfarlane 1994). In the case of *B*. *adolescentis*, three α-glucosidases were identified by Igaue et al. (1983b), but these enzymes did not show any activity against trehalose. AglA has activity against trehalose (Table 2) and seems to be a different enzyme. The degradation of the various substrates by AglB shows similarities with α-glucosidase IIa and IIb (Igaue et al. 1983b). The molecular mass of α-glucosidases IIa and IIb was 60 kDa as estimated by SDS-PAGE and 120 kDa with Sephadex G-200 gel filtration, indicating that these enzymes appear as dimers. The molecular mass estimated by SDS-PAGE for AglB was slightly higher (73 kDa) and the native molecular weight was 149 kDa. Thus, this also forms a dimer. The pH-optimum and temperature optimum curves for α-glucosidase IIa and IIb for maltitol were similar to those for AglB when *p*-nitrophenyl-α-D-glucopyranoside was used as substrate (data not shown). It is concluded that α-glucosidase IIa and IIb, which are probably isoforms, and AglB are the same enzyme.

In *B*. *pseudolongum*, two α-glucosidases were found and only one form, with a molecular weight of 126 kDa estimated by SDS-PAGE and Sephadex G150 gel filtration chromatography, retained activity after partial purification (Degnan and Macfarlane 1994). According to the molecular mass obtained by SDS-PAGE, there is no similarity with this enzyme and AglA and AglB.

No α-amylase activity was detected when the genomic library was plated on LB agar plates with IPTG and AZCL-amylose as substrate. In contrast to this observation, the degradation of starch by *B*. *adolescentis* (Wang et al. 1999) and the purification of an α-amylase form *B*. *adolescentis* (Lee et al. 1997) have been reported. The absence of α-amylase can be due to the fact (i) the α-amylase gene is not expressed although the genomic DNA used for library construction was only partially digested, (ii) no functional α-amylase gene is present in the library or (iii) the α-amylase is not active in *E*. *coli*.

Both α-glucosidases catalyze two types of reactions: hydrolysis and transglucosylation. Hydrolysis is the transfer of a glucosyl residue from the non-reducing end of an oligosaccharide onto water. Transfer of the glucosyl residue onto an acceptor molecule is a transglucosylation reaction (Krasikov et al. 2001). There is a correlation between the hydrolytic substrate specificity and the regioselectivity of the transglucosylation reaction. A specific α-glucosidase from *Bac*.

stearothermophlis, which preferentially hydrolyzes the α(1,4) linkages, showed a preferential transfer to C4 of maltose. Whereas a yeast α-glucosidase with a broader substrate specificity catalyzed the formation of α(1,3), α(1,4), and α(1,6) linkages (Malá et al. 1999). AglA is more specific for α(1,6) linkages and AglB showed a broader substrate specificity (Table 2). The enzymes produced different products and had a different ratio of synthesized oligosaccharides with sucrose as substrate (Figure 2C). Nakao et al. (1994) purified an α-glucosidase of *Bacillus* sp. SAM1606 with the ability to synthesize oligosaccharides from sucrose. One of the oligosaccharides formed was theanderose, a trisaccharide found specifically in honey. A recent study (Kajiwara et al. 2002) showed that the growth and acid production of *Bifidobacterium* spp. was enhanced by honey in a manner similar to that of commercial prebiotics like fructo-oligosaccharides, galacto-oligosaccharides, and inulin. It was assumed that the oligosaccharides present in honey were responsible for this effect. In addition, various transglucosylated oligosaccharides can serve as growth-promoting factors for intestinal bacteria such as bifidobacteria (Shimokawa et al. 1993). The structure of the synthesized oligosaccharides produced by AglA and AglB have yet to be elucidated. Also the transglucosylation reactions have to be optimized for an increased production of these oligosaccharides. The oligosaccharides can be tested for their potential bifidogenic growth effect and/or as prebiotic.

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Chapter 4

Physico-chemical and transglucosylation properties of recombinant sucrose phosphorylase from *Bifidobacterium adolescentis* DSM20083

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ABSTRACT

Clones of a genomic library of *Bifidobacterium adolescentis* were grown in minimal medium with sucrose as sole carbon source. An enzymatic fructose dehydrogenase assay was used to identify sucrose-degrading enzymes. Plasmids were isolated from the positive colonies and sequence analysis revealed that two types of inserts were present, which only differed with respect to their orientation in the plasmid. An open reading frame of 1,515 nucleotides with high homology for sucrose phosphorylases was detected on these inserts. The gene was designated *sucP* and encodes a protein of 56,189 Da. *SucP* was heterologously expressed in *Escherichia coli*, purified, and characterized. The molecular mass of SucP was 58 kDa as estimated by SDS-PAGE, while 129 kDa was found with gel permeation chromatography, suggesting that the native enzyme is a dimer. The enzyme showed high activity towards sucrose and to a lower extent towards α-glucose 1-phosphate. The transglucosylation properties were investigated using a broad range of monomeric sugars as acceptor substrate for the recombinant enzyme, while α-glucose 1-phosphate served as donor. D- and L-arabinose, D- and L-arabitol, and xylitol showed the highest production of transglucosylation products. The investigated disaccharides and trisaccharides were not suitable as acceptor. The structure of the transglucosylation product obtained with D-arabinose as acceptor was elucidated by NMR. The structure of the synthesized non-reducing dimer was α-Glc*p*(1→1)β-Ara*f*.

INTRODUCTION

Three main classes of enzyme are involved in the formation and cleavage of glycosyl linkages. The class of hydrolytic enzymes is the most studied. Another class represents the synthesizing enzymes (glycosyl-nucleotide glycosyltranferases; Kitaoka and Hayashi 2002). The phosphorolytic enzymes are the third class and they catalyze an exo-wise phosphorolysis at the non-reducing end of the glycosyl residues. In most cases, glucosyl residues are phosphorolyzed (Derensy-Dron et al. 1999; Kitaoka and Hayashi 2002), although the phosphorolysis of galactosyl residues (Derensy-Dron et al. 1999) and *N*-acetylglucosaminyl residues (Park et al. 2000) was also recently reported.

Sucrose phosphorylases (EC 2.4.1.7) are members of the phosphorylases and belong to family 13 of the glycoside hydrolases (Henrissat 1991). This type of enzyme, first discovered by Kagan et al. (1942), reversibly catalyses the phosphorolysis of sucrose to α-D-glucose 1-phosphate (G-1-P) and D-fructose. Thus far only the bacterial sucrose phosphorylases produced by *Pseudomonas saccharophila* (Silverstein et al. 1967), *Leuconostoc mesenteroides* (Kawasaki et al. 1996a; Koga et al. 1991), and *Streptococcus mutans* (Russel et al. 1988) have been purified to homogeneity and characterized. Genes for sucrose phosphorylases were cloned from *L. mesenteroides* spp. (Kawasaki et al. 1996b; Kitao and Nakano 1992), *S. mutans* (Ferretti et al. 1988), and *Agrobacterium vitis* (Fournier et al. 1994). Sucrose phosphorylase from *L. mesenteroides* was used to transfer the glucosyl moiety of G-1-P to various sugars and sugar alcohols (Kitao and Sekine 1992; Kitao et al. 1994). Kitao and co-workers showed that the *L. mesenteroides* enzyme also transfers the glucosyl moiety of sucrose to phenolic or alcoholic –OH groups of various substances and showed thereby a broad acceptor specificity (Kitao and Sekine 1994a; 1994b; Kitao et al. 1993; 1995; 2000). Apart from its transglucosylation activity the enzyme can be applied for the determination of inorganic phosphate (Tedokon et al. 1992). Sucrose phosphorylase is also used in an enzymatic sucrose assay (Birnberg and Brenner 1984) and for determination of sucrose with a flow-injection system (Kogure et al. 1997; Maestre et al. 2001). Further, the enzyme can be applied in a one-pot enzymatic galactosyltransferase reaction for the generation of G-1-P in situ (Ichikawa et al. 1995).

Bifidobacteria, a major group of saccharolytic bacteria in the colon, have biological activities related to host health. To increase the number of beneficial bacteria in the gut, the concept of probiotics and/or prebiotics can be used. Non-digestible oligosaccharides can act as a prebiotic (Gibson and Roberfroid 1995). For example, these oligosaccharides can be obtained from plants by

extraction, chemical condensation, or controlled hydrolysis of polysaccharides. Also, transglycosylation reactions can be used for the production of prebiotic non-digestible oligosaccharides (Van Laere et al. 2000). Phosphorylases show transglycosylation properties and can possibly be used for the production of biologically active oligosaccharides. However, for *Bifidobacterium* spp. only a β-1,3-galactosyl-*N*-acetylhexosamine phosphorylase has been partially purified and characterized. This enzyme from *Bifidobacterium bifidum* (Derensy-Dron et al. 1999) was able to synthesize Galβ1→3GlcNAc derivatives with α-D-Gal*p*-1-P5 as donor and different Glc*p*NAc derivatives as acceptor (Farkas et al. 2000). Recently, sucrose-utilizing genes from *B. lactis*, containing a sucrose phosphorylase, a transcriptional regulator, and a sucrose transporter, were described. The genes were similarly induced by sucrose and raffinose but repressed by glucose (Trindade et al. 2003).

Here we report on the identification of a sucrose phosphorylase (*sucP*) gene from *B. adolescentis* and the physico-chemical and transglucosylation properties of the recombinant sucrose phosphorylase. The enzyme was identified by screening a genomic library (chapter 2; Van den Broek et al. 1999) and its sucrose degrading activity was detected with a fructose dehydrogenase assay (Holmes 1997).

MATERIALS AND METHODS

Genomic library and screening. The genomic library of *B. adolescentis* DSM20083 was constructed as described in chapter 2 (Van den Broek et al. 1999). The genomic library was spread on large square LB agar plates containing 1 mM iso-propyl *β*-D-thiogalactopyranoside (IPTG), 32 mg X-gal l^{-1} and 100 mg carbenicillin l^{-1}. Plates were incubated at 37 ^{o}C for about 24 h. White colonies were randomly picked with the aid of an automated colony picker and transported onto microtiter plates. Each well contained 120 µl of 36 mM K_2HPO_4, 13.2 mM KH_2PO_4, 1.7 mM tri-sodium citrate, 0.4 mM $MgSO_4$, 0.9 g $(NH_4)_2SO_4$ l^{-1}, 44 g glycerol l^{-1}, 10 g trypton l^{-1}, 5 g yeast extract l^{-1}, and 5 g NaCl l^{-1}. Plates were incubated overnight at 37 ^{o}C and stored at –80 ^{o}C. For the determination of sucrose-degrading activity, duplicate plates were made, in which each well contained 120 µl LB broth supplemented with 1 mM IPTG and 50 mg ampicillin l^{-1}. Plates were incubated for 48 h at 37 ^{o}C. After incubation, 100 µl H_2O was added and the plates were centrifuged for 10 minutes at 1,500 *g*. The supernatants were used in a fructose dehydrogenase

assay for sucrose-degrading activity, according to Holmes (1997). Enzyme solution (50 µl) was incubated with 0.03 units fructose dehydrogenase, 10 µl phenazine methosulfate (0.6 g l^{-1}), 20 µl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 0.6 g l^{-1}), and 110 µl 3 mM sucrose. Incubation was performed for 1 h at 37 ^{o}C and a positive reaction was visualized by the development of a purple color.

Isolation and characterization of recombinant sucrose phosphorylase. *Escherichia coli* cell culture (1 l) containing the recombinant SucP was grown overnight and centrifuged for 10 min at 8,000 *g* at 4 °C. The supernatant was filtered through a 0.2-µm filter and dialyzed overnight against 20 mM piperazine (pH 6.5) at 4 °C. Cells were suspended in 50 ml 20 mM piperazine (pH 6.5) and disrupted by sonic treatment (10 min; 30% duty cycle; Branson Sonifier 250) on ice. The suspension was centrifuged for 15 min at 20,000 *g* at 4 °C and the supernatant was collected. The pellet was suspended in 40 ml 20 mM piperazine (pH 6.5) and sonicated as described above. This treatment was repeated once. The supernatants were pooled and used for further purification as cell-free extract.

Both enzyme preparations were purified on a Q-Sepharose (Amersham) anion exchange column. After application, elution took place with a linear gradient of 0–1 M NaCl in 20 mM piperazine (pH 6.5) at a flow rate of 57 cm h^{-1}. Fractions with the highest sucrose phosphorylase activity were pooled and further purified on a Superdex 75 PG (Amersham) gel permeation column. Elution was performed with 0.15 M NaCl in 20 mM piperazine (pH 6.5) at a flow rate of 30 cm h^{-1}. Fractions with the highest enzyme activity were pooled and named “cell culture supernatant extract” and “cell-free extract”, respectively.

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

SDS-PAGE was carried out on the Pharmacia PhastSystem according to the instructions of the supplier. Coomassie brilliant blue staining was used for detection of proteins on the PhastGel 10-15% gradient gels (Amersham). The native molecular mass was determined by gel exclusion chromatography using the Äkta Purifier equipped with a Superdex 200 HR column (Amersham). The column was calibrated with the high- and low-molecular mass standards for gel permeation (Amersham). Elution was performed with 20 mM piperazine buffer (pH 6.5) containing 0.15 M NaCl at a flow rate of 38 cm h^{-1}.

The N-terminal amino acid sequence was determined at the Sequence Center of Utrecht University (Utrecht, The Netherlands) and was performed as described in chapter 3 (Van den Broek

et al. 2003). For Southern blot analysis, the coding part of *SucP* was amplified by PCR with primer P5For (5′-ATGAAAAACAAGGTGCAGCTC-3′) and primer P3Rev (5′-TCAGGCGACGACAGGCGGATT-3′). Chromosomal DNA from *B. adolescentis* was digested with *Hinc*II (Fermentas). The analysis and detection was the same as described in chapter 3 (Van den Broek et al. 2003).

Enzyme assays. Sucrose phosphorylase was assayed by measuring the rate of reduction of $NADP^+$ in a coupled system containing sucrose phosphorylase, phosphoglucomutase, and glucose-6-phosphate dehydrogenase (Koga et al. 1991; Silverstein et al. 1967). The standard assay contained 50 mM potassium phosphate buffer (pH 6.8), 0.14 M sucrose, 0.09 mM EDTA-Na_2, 0.36 mM $NADP^+$, 0.003 mM D-glucose-1,6-diphosphate, 15 mM $MgCl_2$, 6 units α-phosphoglucomutase ml^{-1}, and 6 units glucose-6-phosphate dehydrogenase ml^{-1}. The increase in absorbance at 340 nm was recorded in a spectrophotometer thermostatted at 30 ^oC. One unit of sucrose phosphorylase activity was defined as the amount of enzyme that reduced 1 μmol of $NADP^+$ min^{-1}. A molar extinction coefficient of 6,220 M^{-1} cm^{-1} was used for calculating the enzyme activity (Koga et al. 1991).

The activity of SucP towards different substrates, with inorganic phosphate as acceptor, was measured in 50 mM potassium phosphate buffer (pH 6.8) and 0.14 M substrate at 30 ^oC for 2 h. The final concentration of enzyme was 3 units ml^{-1}. The reaction was stopped at 100 ^oC for 10 min. After centrifugation for 10 min at 10,000 *g*, the supernatant was analyzed by high-performance anion exchange chromatography (HPAEC), using a CarboPac PA-100 or PA-1 (4 x 250 mm; Dionex) column and a pulsed electrochemical detector (Dionex) in pulsed amperometric detection mode. A flow rate of 1 ml min^{-1} was used with the following gradient of sodium acetate in 0.1 M NaOH: 0–15 min at 0–420 mM, 15.0–15.1 min at 420-1,000 mM, 15.1–20 min at 1,000 mM.

Temperature and pH optima were determined under the same conditions as above, measuring the release of glucose from sucrose (0.14 M). For the temperature optimum, a range of 4–80 ^oC was used. For the pH optimum, the potassium phosphate buffer was in the range of pH 5–8.5.

Acceptor specificity of SucP with G-1-P as donor was measured in 20 mM piperazine buffer (pH 6.5), 0.9 M acceptor, and 0.9 M G-1-P at 30 ^oC for 6 h. The final concentration of enzyme added was 10 units ml^{-1}. A product progression profile with L-arabinose or L-sorbose as acceptor was in the range of 0–8 h. The reaction was stopped and analyzed as described above. The following gradient of sodium acetate in 0.1 M NaOH was used: 0–10 min at 0 mM, 10–25 min at 0–420 mM, 25.0–25.1 min at 420–1,000 mM, 25.1–30.0 min at 1,000 mM.

Cellobiose was obtained from BDH, 1-kestose from Megazyme, and D-arabinose, L-arabinose, D-galactose, D-glucose, D-fructose, lactose, maltose, D-mannose D-sorbitol, sucrose, D-xylose, and $MgCl_2$ from Merck. Rhamnose was from Janssen and all other chemicals and enzymes were from Sigma.

Sucrose phosphorylase activity in *B. adolescentis*. *B. adolescentis* DSM20083 was grown anaerobically overnight at 37 ^{o}C in MRS medium (pH 6.0) containing 0.5 g cysteine l^{-1}. Cells were washed once in 50 mM potassium phosphate buffer (pH 6.8). After centrifugation for 10 min at 1,500 *g*, cells were suspended in the phosphate buffer and then divided into different media in duplicate. Medium A consisted of MRS medium with cysteine, medium B, C, and D contained a defined medium with, respectively, glucose or sucrose as carbon source or no sugar added. The defined medium (Kabel et al. 2003) was prepared by combining solutions S1, S2, and S3 in a ratio (by volume) of 17:1:2. S1 contained 60 g glucose l^{-1}, sucrose, or only water. S2 contained 134 g Bacto yeast nitrogen base l^{-1} (Difco). S3 contained $MgSO_4$ (0.2 g l^{-1}), $CaCl_2$ (0.2 g l^{-1}), K_2HPO_4 (1 g l^{-1}), KH_2PO_4 (1 g l^{-1}), $NaHCO_3$ (10 g l^{-1}), NaCl (2 g l^{-1}), casein enzymatic hydrolysate (Difco; 50 g l^{-1}), and sodium thioglycolate (Sigma; 5 g l^{-1}). The inoculated media (50 ml) were incubated anaerobically at 37 ^{o}C for 24 h. Cells were washed two times with 25 ml potassium phosphate buffer (pH 6.8). After the last washing step cells were suspended in 10–15 ml 50 mM potassium phosphate buffer (pH 6.8) and disrupted by sonic treatment (10 min; 30% duty cycle; Branson Sonifier 250) on ice. The suspension was centrifuged for 15 min at 20,000 *g* at 4 °C and the supernatant was collected and analyzed for sucrose phosphorylase activity (Koga et al. 1991; Silverstein et al. 1967) and protein content.

Structure elucidation of transglucosylation product. The reaction mixture with D-arabinose as acceptor was applied to a Bio-Gel P2 (Biorad Laboratories) size exclusion column, to isolate the transglucosylation product. Elution took place with Millipore water of 60 ^{o}C at a flow rate of 5.7 cm h^{-1}. Sugars were detected with a SHODEX RI 72 refractive index refractometer (Showa Denko). Fractions were also analyzed by HPAEC and fractions containing the transglucosylation product were pooled. The pooled fraction was exchanged three times in 99.96% D_2O and after the last freeze drying step dissolved in 99.996 % D_2O (Cambridge Isotope Laboratories). NMR spectra were recorded at a probe temperature of 70 ^{o}C on a Bruker AMX-500 spectrometer located at the Wageningen NMR Centre. The NMR machine was operated at 500.13 MHz for ^{1}H and 125.77 MHz for ^{13}C; and chemical shifts were expressed relative to an internal acetone standard: 2.225 ppm for ^{1}H and 31.55 ppm for ^{13}C. All 1D ^{1}H and ^{13}C, 2D correlation (COSY), and 2D HMBC homo- and heteronuclear NMR experiments were performed according to Verhoef et al. (2002).

DNA sequencing and sequence analysis. An automated DNA Sequencer 373 (Applied Biosystems) was used for determining the nucleotide sequence of the genes. The DNA sequence data were submitted to the GenBank Nucleotide Databases under the accession number AF543301. The BLAST2 program (Altschul et al. 1997) was used for searching sequence homologies.

RESULTS

Identification of sucrose phosphorylase gene from *B. adolescentis*. A genomic library was screened for sucrose-degrading enzyme activity, using an enzymatic fructose assay. From the positive colonies, plasmids were isolated and restriction analysis was performed. Two types of restriction pattern were found and both inserts had a size of 2.4 kb. Sequence analysis revealed that both inserts were the same and only differed with respect to the orientation of the insert in the plasmid. An open reading frame (ORF) of 1,515 nucleotides was detected on these inserts. A putative ribosome-binding site was found upstream of the initiation codon of the ORF. The deduced amino acid sequence contained 504 amino acids, encoding a protein of 56,189 Da. A database search revealed 92, 85, 55, and 55% identities, respectively, with sucrose phosphorylases or sucrose phosphorylase-like proteins from *B. longum* (AE014674, AY236071), *B. lactis* (AF441242), *A. tumefaciens* (AF065245), and *A. vitis* (Z22732, Z22733, Z22734). The gene was designated *SucP*. All these sucrose phosphorylases are members of family 13 of the glycoside hydrolases (Henrissat 1991) and therefore SucP was also classified within this family. Southern blot analysis of *SucP* with digested chromosomal DNA from *B. adolescentis* confirmed the presence of the gene in *B. adolescentis*. A fragment at around 700 bp strongly hybridized with the probe, which corresponded with the internal fragment of 699 bp obtained by digestion of the *SucP* gene with *Hinc*II. Two weaker signals were observed at 500 bp and 800 bp and the corresponding fragments were overlapping just the beginning or the end of the *SucP* gene, respectively. Just below 100 bp faint signals corresponded with the two small internal fragments of 81 and 63 bp.

Promoter region of *SucP*. IPTG was added to express the proteins under control of the *lac* promoter of the *p*Bluecript phagemid. However, the addition of IPTG did not increase the expression level of the enzyme. In addition, both plasmids, with different orientations of their inserts, showed the production of recombinant SucP. These results show that the expression of SucP is under control of the original gene promoter. Figure 1 shows the multiple nucleotide sequence

alignment of the promoter regions of sucrose phosphorylase from *B. adolescentis* (AF543301), *B. lactis* (AF441242), and *B. longum* (AY236071, AE014674). Apart from the high homology for the sucrose phosphorylase gene (data not completely shown), three coding parts in the promoter region where highly conserved. The first region was a potential Shine-Dalgarno sequence at position 5, relative to the ATG start codon. For *B. lactis*, Trindade et al. (2003) found a transcription start site for sucrose phosphorylase at position 102 of the initiation code, followed by an inverted repeat that could serve as a potential operator for a sucrose phosphorylase regulator. This region was also highly conserved in *B. adolescentis* and *B. longum*. The transcription site for *B. adolescentis* was probably at position 88 of the ATG start codon and for *B. longum* at position 102. A GalR-LacI-type transcriptional regulator gene was found in the opposite direction to the sucrose phosphorylase gene from *B. lactis*. In *B. longum* SJ32 (AY236071), a putative sucrose regulator was also present in the opposite direction of the sucrose phosphorylase gene. Comparison of these sucrose regulator genes revealed only 25% identity. For *B. adolescenti*s, no ORF or partial coding parts in the nucleotide sequence (876 bp) preceding *SucP* could be identified for such a regulator gene. The third highly conserved region was located between position 164 and 135 and showed some homology with the inverted repeat of region 2. No putative promoter −35 and −10 regions, as identified for *B. lactis*, could be found for *B. adolescentis* and *B. longum*.

B. adolescentis was grown in MRS medium and defined medium with either glucose or sucrose as carbon source, or no sugar added. The optical densities at 600 nm (OD_{600}) after 24 h growth were 1.302±0.001, 0.646±0.013, and 0.119±0.007, respectively. When no sugar was added, no growth was observed. The sucrose phosphorylase specific activities of the cell-free extracts from MRS medium and defined medium with either glucose or sucrose were 0.15±0.01, 0.05±0.01, and 0.84±0.00 units mg^{-1}, respectively. *B. adolescentis* was able to grow in defined medium with sucrose as sole carbon source. However, the growth rate was very low. Sucrose phosphorylase activity was also present when cultured in MRS medium.

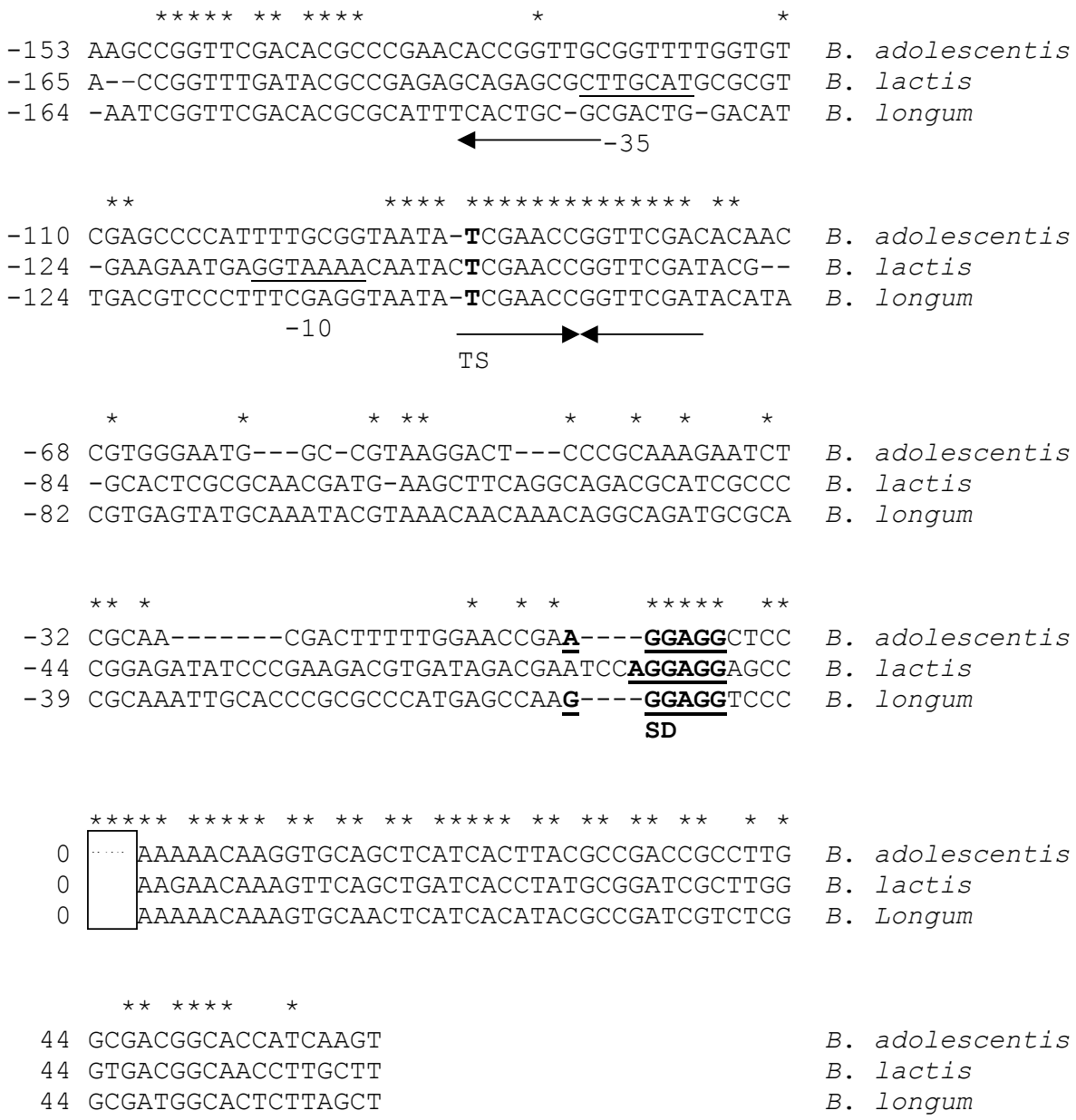

Figure 1: Multiple-nucleotide sequence alignment by the Clustal method of the promoter region of sucrose phosphorylase from *Bifidobacterium adolescentis* (AF543301), *B. lactis* (AF441242), and *B. longum* (AY236071, AE014674). The ATG start codon is *boxed* and the predicted ribosome-binding site or potential Shine-Dalgarno sequence (*SD*) are *in bold and underlined*. The transcriptional start site is indicated *in bold* (TS) and *the asterisks* indicate identical nucleotides. *The arrows* indicate the direction of the direct and indirect repeat sequences of *B. lactis* and the putative promoters are *underlined* (–35 and –10; Trindade et al. 2003).

Purification and characterization of SucP. The cell-free extract of 1 l cell culture contained 19,357 units enzyme activity, while the cell culture supernatant contained 6,340 units. Both enzyme preparations were used for further purification. After anion exchange chromatography and gel permeation chromatography, the enzyme appeared to be pure, as judged by SDS-PAGE and Coomassie brilliant blue staining. The purified enzyme from the cell culture supernatant showed a slightly higher specific activity (109 U mg^{-1}) than the enzyme purified from the cell-free extract (85 U mg^{-1}). Therefore, sucrose phosphorylase from the cell culture supernatant was used for further study. Some physico-chemical properties of the recombinant sucrose phosphorylase are given in Table 1. The molecular mass estimated by SDS-PAGE was in agreement with the molecular mass calculated from the deduced amino acid sequence. Native molecular mass determination indicated that SucP is a dimer. The N-terminal amino acid sequence of SucP from both the cell culture supernatant and the cell-free extract was MKNKVQLITYADRLG; and this is identical to the N-terminus of the deduced amino acid sequence of *SucP*.

TABLE 1: Physico-chemical properties of recombinant sucrose phosphorylase from *Bifidobacterium adolescentis*

Parameter	SucP value
Specific activity towards sucrose	109 units mg^{-1}
Deduced molecular mass	56,189 Da
Molecular mass (SDS-PAGE)	58 kDa
Native molecular mass (gel permeation)	129 kDa
Optimum pH	pH 6–6.5
Optimum temperature	48 ^{o}C

Hydrolyzing and transglucosylation properties of SucP. In the presence of inorganic phosphate, SucP was only active towards sucrose (phosphorolysis) and to a lesser extent G-1-P (hydrolysis). No activity was observed with other substrates containing glucose moieties (Table 2). The transglucosylation reaction was studied with G-1-P as donor and different acceptors. The formation of new products was followed over time for L-arabinose and L-sorbose as acceptor. Within 1 h, most of the synthesized product (>80%) was formed. Under the conditions used, maximal production was reached after 4 h for these acceptors. Based on these results, experiments with different acceptors were performed, using a reaction time of 6 h. Using disaccharides and

trisaccharides as acceptor, no new transglucosylation products were formed (Table 3). Most monomeric sugars could be used as acceptor (Table 3). Some examples are given in Figure 2. The highest transglucosylation activity was observed for L-arabinose, L-arabitol, and D-xylitol. In the case of D-xylitol, two products were synthesized instead of one product, as found for the other monomeric sugar acceptors (Figure 2C). α-Methyl-D-glucose inhibited the hydrolysis of G-1-P, because a smaller amount of G-1-P was converted, in comparison with the other incubation mixtures without transglucosylation activity. This was also observed with D-glucose as acceptor. However, the equilibrium between glucose, inorganic phosphate and G-1-P could be influenced by higher amounts of D-glucose in the reaction mixture.

TABLE 2: Enzyme activity towards different substrates with inorganic phosphate as acceptor for recombinant sucrose phosphorylase from *Bifidobacterium adolescentis*. Activity was determined by high performance anion exchange chromatography (HPAEC), as described in Materials and methods. Each activity was compared with the enzyme activity towards sucrose as substrate (scale ++ equal, + less, – no yield)

Substrate (type of linkage)	Activity
Sucrose (α-glc-1,2 *p*βfru)	++
α-D-Glucose-1-phosphate	+
α-D-Galactose-1-phosphate	–
α-D-Glucose-6-phosphate	–
β-D-Glucose-1-phosphate	–
D-fructose-1-phosphate	–
Cellobiose (β-glc-1,4)	–
D-Glucose	–
1-Kestose (α-glc-1,2 *p*βfru-1,2 *p*βfru)	–
Lactose (β-gal-1,4 glc)	–
Maltose (α-glc-1,4)	–
Melezitose (α-glc-1,2 *p*βfru-3,1 α-glc)	–
Palatinose (α-glc-1,6 *p*βfru)	–
Raffinose (α-gal-1,6 α-glc-1,2 *p*βfru)	–
Trehalose (α-glc-1,1)	–

TABLE 3: Acceptor specificity of recombinant sucrose phosphorylase from *Bifidobacterium adolescentis* with α-glucose 1-phosphate (G-1-P) as donor. Activity was determined by HPAEC, as described in the Materials and methods. Scale: – no increase; + less; ++ equal; +++ increased amount of transglucosylation products formed in comparison with D-arabinose as acceptor. The decrease in G-1-P was used to estimate the amount of transglucosylation product synthesized

Acceptor	Transglucosylation activity
D-Arabinose	++
L-Arabinose	+++
D-Arabitol	++
L-Arabitol	+++
D-Fucose	+
L-Fucose	++
D-Fructose	+
D-Galactose	+
D-Glucose	–
L-Glucose	+
α-Methyl-D-glucose	–
D-Mannose	–
L-Mannose	–
L-Rhamnose	–
D-Sorbitol	++
L-Sorbose	++
D-Xylitol	+++
D-Xylose	+
L-Xylose	+
Cellobiose	–
Lactose	–
Lactulose	–
Maltitol	–
Maltose	–
Maltotriose	–

Melezitose	–
Melibiose	–
Raffinose	–
Sucrose	–
Trehalose	–

Transglucosylation product elucidation. The transglucosylation product obtained with D-arabinose as acceptor (Figure 2A) was purified from the reaction mixture by gel exclusion chromatography. From 1D ^{1}H proton NMR spectrum, two anomeric signals can be seen at 5.24 ppm ($J_{(1-2)}$=4.59 Hz) and 5.13 ppm ($J_{(1-2)}$=3.31 Hz) with equal intensity (Figure 3). The fact that no α/β-anomerization of the oligomer can be seen indicates that no reducing-end is present and the two sugar moieties are (1→1) linked. A 2D homonuclear COSY experiment made it possible to assign all proton chemical shifts of the two sugar residues. The signal at 5.24 ppm belongs to the β-arabinose, whereas the signal at 5.13 ppm belongs to the α-Glc moiety. To reveal the linkage type of the oligosaccharide, a 2D heteronuclear HMBC spectrum was recorded. Using this spectrum the ^{13}C chemical shifts, pyranose/furanose conformation and linkage type could be determined. Table 4 shows the ^{1}H and ^{13}C chemical shifts of both sugar residues. The ^{13}C chemicals shifts were compared with published data, confirming that residue A is a β-arabinose and residue B is α-glucose (Bock and Thøgersen 1982; Bock et al. 1984).

TABLE 4: ^{1}H and ^{13}C chemical shifts in ppm of the transglucosylation product, obtained with D-arabinose as acceptor and α-glucose 1-phosphate as donor by recombinant sucrose phosphorylase from *Bifidobacterium adolescentis*. Chemical shifts are expressed relative to an internal acetone standard: ^{1}H= 2.225 ppm, ^{13}C=31.55 ppm

Residue	H-1 (C-1)	H-2 (C-2)	H-3 (C-3)	H-4 (C-4)	H5/5' (C-5)	H-6/6' (C-6)
β-Ara*f*	5.24	4.19	4.75	3.88	3.79/3.70	
	(103.38)	(78.08)	(75.78)	(82.23)	(64.46)	
α-Glc*p*	5.12	3.58	3.74	3.48	3.86	3.77/3.84
	(100.80)	(72.93)	(74.38)	(70.99)	(74.08)	(61.94)

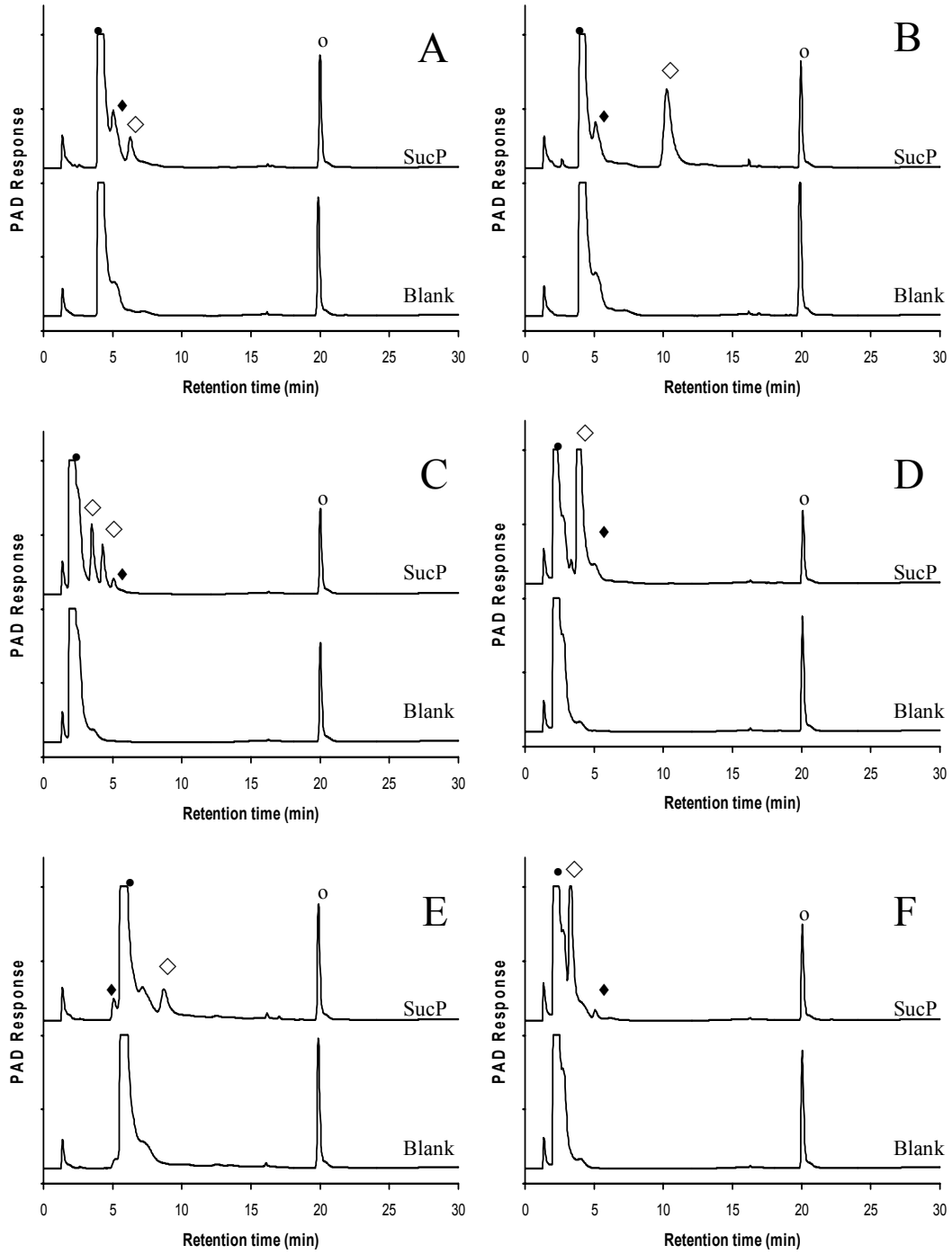

Figure 2: High performance anion exchange chromatography of reaction products formed with D-arabinose (**A**), L-arabinose (**B**), D-xylitol (**C**), L-arbitol (**D**), L-sorbose (**E**), and D-arbitol (**F**) as acceptor and α-glucose 1-phosphate as donor by recombinant sucrose phosphorylase from *Bifidobacterium adolescentis*. *Black circles* Acceptor, *white circles* α-glucose 1-phosphate, *white diamonds* transglucosylation product(s), *black diamonds* glucose.

The β-Ara residue was found to be in the furanose conformation, which could be proven by the intense Ara C-1, Ara H-4 (Figure 3) and Ara C-4, Ara H-1 cross-peaks (data not shown). The ^{13}C chemical shift of C-4 at 83.23 ppm shows a typical 10 ppm down-field shift when compared to its pyranose conformation (Bock and Thøgersen 1982). The glycosidic linkage of the dimer is clearly α-Glc*p*(1→1)β-Ara*f* which was demonstrated by the inter-residual scalar coupling between Ara C1, Glc H-1 and Glc C-1, Ara H-1 (Figure 3). All the NMR experiments together showed that the structure of the transglucosylation product formed was a α-Glc*p*(1→1)β-Ara*f* dimer.

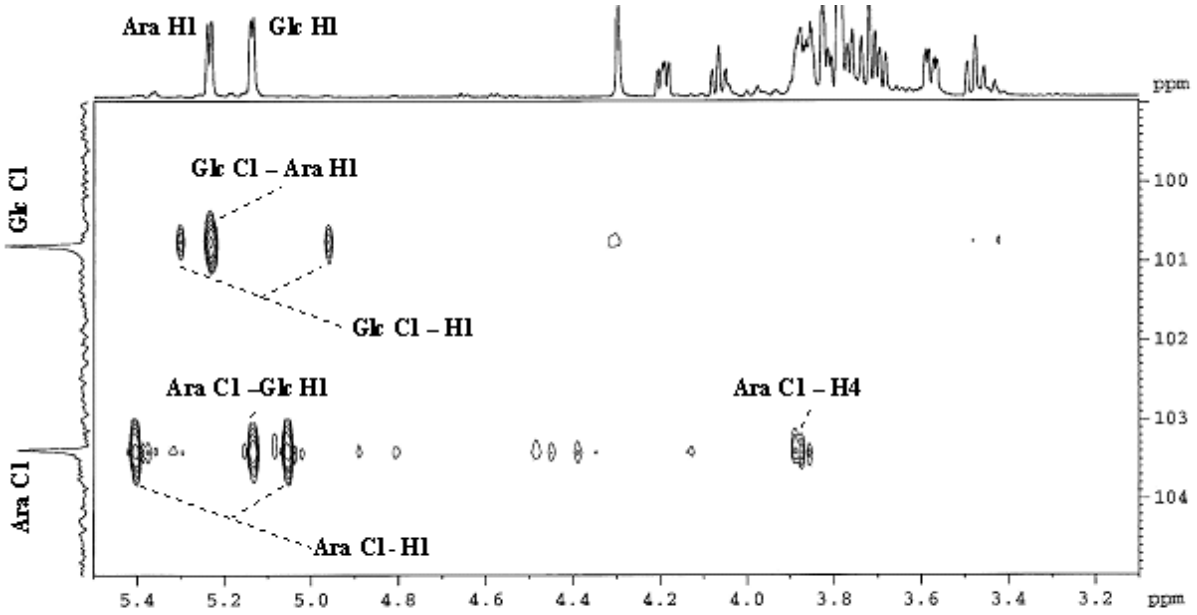

Figure 3: 500-MHz 2D ^{1}H ^{13}C undecoupled HMBC spectrum of the transglucosylation product, obtained with D-arabinose as acceptor and α-glucose 1-phosphate as donor by recombinant sucrose phosphorylase from *Bifidobacterium adolescentis*, recorded by NMR in D_2O at 70 ^{o}C. The spectrum shows the 1D ^{1}H-spectrum and the anomeric region of the ^{13}C spectrum as *top and left projection*, respectively.

DISCUSSION

Screening a genomic library of *B. adolescentis* with an enzymatic assay based on fructose dehydrogenase resulted in the identification of a sucrose phosphorylase gene (*sucP*). The fructose dehydrogenase assay was developed for the enzymatic determination of sucrose in serum and urine (Holmes 1997). Sucrose is converted with an invertase into D-fructose and D-glucose. Subsequently, fructose dehydrogenase converts D-fructose and MTT with phenazine methosulfate into 5-keto-D-fructose and MTT-formazan, which gives a purple color. The supernatant of the different colonies, grown in minimal medium with sucrose as sole carbon source, were incubated for sucrose-degrading enzymes. The enzymes able to release fructose from sucrose are α-glucosidases, sucrose phosphorylases, invertases, and β-fructofuranosidases. Using the fructose dehydrogenase assay, only a sucrose phosphorylase gene was identified. In chapter 3 two α-glucosidase genes were identified from *B. adolescentis* DSM20083 (Van den Broek et al. 2003). AglB was able to degrade sucrose, whereas high amounts of AlgA were needed to observe any degradation of sucrose. A β-fructofuranosidase isolated from *B. adolescentis* G1 was also able to degrade sucrose (Muramatsu et al. 1993). The release of fructose by these enzymes was probably below the detection limit of the enzymatic fructose assay.

The molecular mass of SucP, estimated by SDS-PAGE, is 58 kDa and is almost identical with sucrose phosphorylase from *L. mesenteroides* spp. (between 54–58 kDa; Kawasaki et al. 1996a; Koga et al. 1991) and *S. mutans* (56 kDa; Ferretti et al. 1988). The sucrose phosphorylase from *P. saccharophila* (Silverstein et al. 1987) has a higher molecular mass (87 kDa). However, these enzymes behave as monomeric proteins as estimated by gel permeation, while SucP from *B. adolescentis* appears as a dimer.

Sucrose phosphorylase reversibly catalyzes the phosphorolysis of sucrose to G-1-P and D-fructose. This reaction is energetically advantageous, because it bypasses the ATP-requiring step of the hexokinase reaction to phosphorylate glucose in preparation for glycolysis. Trindade et al. (2003) identified a sucrose utilization gene cluster of *B. lactis* containing a sucrose phosphorylase, a GalR-LacI-type transcriptional regulator, and a sucrose transporter gene. This indicates that bifidobacteria are able to utilize sucrose and can directly form G-1-P. *B. adolescentis* can release sucrose from non-digestible oligosaccharides like stachyose or raffinose by α-galactosidase (Van Laere et al. 1999) or from fructo-oligosaccharides by β-fructofuranosidase (Muramatsu et al. 1993). These non-digestible oligosaccharides are applied as prebiotics.

Sucrose phosphorylase activity, specifically measured by the increase of NADPH in an enzymatic coupled assay, was present in cell-free extracts of *B. adolescentis* grown in MRS medium and in defined medium with glucose or sucrose. Sucrose phosphorylase was highest in defined medium with sucrose; and the lowest activity was observed with glucose as sole carbon source. Trindade et al. (2003) found the lowest sucrase activity with glucose as sole carbon source. In their study the degradation of sucrose was measured by the increase in reducing end-groups. However, the hydrolysis of sucrose can be due to α-glucosidases, β-fructofuranosidases and/or sucrose phosphorylase. In our study, the OD_{600} values were not the same for the different incubations, which can influence a comparison of the specific sucrose phosphorylase activity of the cell-free extracts.

The phosphorolysis reaction of sucrose phosphorylases proceeds via a ping-pong bi-bi mechanism. The formation of a glucose-enzyme complex and the subsequent reaction of this complex with an acceptor, to form the reaction products, are involved in this double-displacement mechanism (Doudoroff et al. 1947a; Silverstein et al. 1967). In addition, the enzymes classified in family 13 of the glycoside hydrolases (Henrissat 1991) are retaining, indicating that they have a double-displacement mechanism. Sucrose phosphorylase has a high substrate specificity for sucrose, and G-1-P. Using these substrates as glucosyl donor, a broad range of glucosyl acceptors can be used (Kitao and Sekine 1992; Kitao et al. 1993; 1995; Weimberg and Doudoroff 1954). A sucrose phosphorylase preparation from dry cells of *P. saccharophila* (Doudoroff et al. 1947b) produced, for example, a high amount of transglycosylation products with L-arabinose, D-fructose, and L-sorbose as acceptor and G-1-P as donor. Gottschalk (1950) proposed that the glucosyl acceptor always has a hydroxyl group in a *cis* position relative to the glucosidic bond. Sucrose phosphorylase from *P. putrifans* was not able to use L-sorbose and D-xyloketose as acceptor (Weimberg and Doudoroff 1953), although the hydroxyl group has the right configuration. The acceptor specificity of sucrose phosphorylase from *L. mesenteroides* was very broad and no regularity for the position of the hydroxyl group was observed (Kitao and Sekine 1992). This was also observed for the recombinant sucrose phosphorylase from *B. adolescentis* in this study. Our findings imply that it is not always necessary that the hydroxyl group of the glucosyl acceptor to be at the *cis* position relative to the glucosidic bond. The 3D characterization of SucP is described in chapter 5 (Sprogøe et al. 2004).

Using arabinose, arabitol, and xylitol as acceptors resulted in a high yield of transglucosylation product. A crude extract from *P. saccharophila* was able to synthesize the disaccharide α-D-glucopyranosyl-(1-3)-L-arabinofuranoside from L-arbinose (Doudoroff et al.

1947b). Sucrose phosphorylase from *L. mesenteroides* and xylitol as acceptor formed α-D-glucopyranosyl-(1-4)-xylitol (Kitao and Sekine 1992). For SucP and xylitol as acceptor, two new peaks were detected by HPAEC (Figure 2C), which implies that more than one product was formed. Kojibiose and nigerose were formed with sucrose as acceptor by *L. mesenteroides* (Kitao et al. 1994). These products were not detected when SucP was incubated with G-1-P and sucrose. Only the hydrolysis product glucose and fructose were obtained. In addition we found that SucP was able to form a transglucosylation product with D-arabinose as acceptor. Sucrose phosphorylases from other microorganisms are not able to use D-arabinose as acceptor (Doudoroff et al. 1947b; Kitao and Sekine 1992; Weimberg and Doudoroff 1953). The identified structure was a non-reducing dimer which consisted of αGlc*p*(→)β-Ara*f*. Further research is focusing on elucidating the structure of other transglucosylation products. Apart from G-1-P as donor, sucrose can also be investigated as donor molecule for the formation of new products. Oligosaccharide products such as the αGlc*p*(→)β-Ara*f* can be tested for their ability to act as bifidogenic factor and/or prebiotic.

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Chapter 5

Crystal structure of sucrose phosphorylase from *Bifidobacterium adolescentis*

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ABSTRACT

Around 80 enzymes are implicated in the generic starch and sucrose pathways. One of these enzymes is sucrose phosphorylase, which reversibly catalyzes the conversion of sucrose and orthophosphate to D-fructose and α-D-glucose 1-phosphate. Here, we present the crystal structure of sucrose phosphorylase from *Bifidobacterium adolescentis* (SucP or BiSP) refined at 1.77 Å resolution. It represents the first 3D structure of a sucrose phosphorylase and is the first structure of a phosphate-dependent enzyme from the glycoside hydrolase family 13. The structure of SucP is composed of the four domains A, B, B′, and C. Domain A comprises the $(\beta/\alpha)_8$-barrel common to family 13. The catalytic active-site residues (Asp192 and Glu232) are located at the tips of β-sheets 4 and 5 in the $(\beta/\alpha)_8$-barrel, as required for family 13 members. The topology of the B′ domain disfavors oligosaccharide binding and reduces the size of the substrate access channel compared to other family 13 members, underlining the role of this domain in modulating the function of these enzymes. It is remarkable that the fold of the C domain is not observed in any other known hydrolases of family 13. SucP was found as a homodimer in the crystal, and a dimer contact surface area of 960 $Å^2$ per monomer was calculated. The majority of the interactions are confined to the two B domains, but interactions between the loop 8 regions of the two barrels are also observed. This results in a large cavity in the dimer, including the entrance to the two active sites.

INTRODUCTION

Bifidobacterium adolescentis forms one of the major groups of bifidobacteria of the large intestine in human adults (Matsuki et al. 1999) and can be used as a probiotic, e.g. in yogurt. Probiotics are used as microbial food supplements to beneficially affect the host by improving its intestinal microbial balance (Gibson and Roberfroid 1995). The interaction of resistant starch with gut flora throughout the digestive tract can also promote human health (Bird et al. 2000). This brings the enzymes involved in starch and α-gluco-oligosaccharides (e.g. sucrose) metabolism of *B. adolescentis* in focus. In chapter 3 two α-glucosidases from *B. adolescentis* were cloned and characterized (Van den Broek et al. 2003), but to our knowledge only a single enzyme from the *Bifidobacterium* genome has been characterized by a 3D structure (Iwata and Ohta 1993).

Around 80 enzymes are implicated in the generic starch and sucrose pathways according to the Kegg database (http://www.genome.ad.jp/kegg/). One of these is the sucrose phosphorylase which reversibly catalyzes the reaction:

sucrose + orthophosphate = D-fructose + α-D-glucose 1-phosphate

This reaction enables the production of the essential glucose moiety from sucrose. The gene coding for sucrose phosphorylase in *B. adolescentis* (*sucP*) has been sequenced (accession no. AF543301), cloned and characterized as described in chapter 4 (Van den Broek et al. 2004). SucP (or BiSP) consists of 504 amino acids resulting in a molecular mass of 56,189 g mol^{-1}. The only other bacterial sucrose phosphorylases purified to homogeneity and characterized today are those produced by *Pseudomonas saccharophila* (Silverstein et al. 1967), *Leuconostoc mesenteroides* (Kawasaki 1996; Koga et al. 1991), and *Streptococcus mutans* (Russel et al. 1988).

On the basis of amino acid sequence similarities, SucP has been placed in the retaining glycoside hydrolase (GH) family 13 (Coutinho and Henrissat 1999), also called the α-amylase family. Some members show transglycosidase activity, e.g. cyclomaltodextrin glucanotransferase and amylosucrase (AS). Structurally, the GH family 13 is characterized by having a $(\beta/\alpha)_8$-barrel comprising the catalytic domain and referred to as domain A. Apart from the catalytic domain, the enzymes of the family typically contain several other domains. These are named the N (N-terminal domain), B (a domain formed by the usually large loop 2 in the $(\beta/\alpha)_8$-barrel), and the C domain (C-terminal domain).

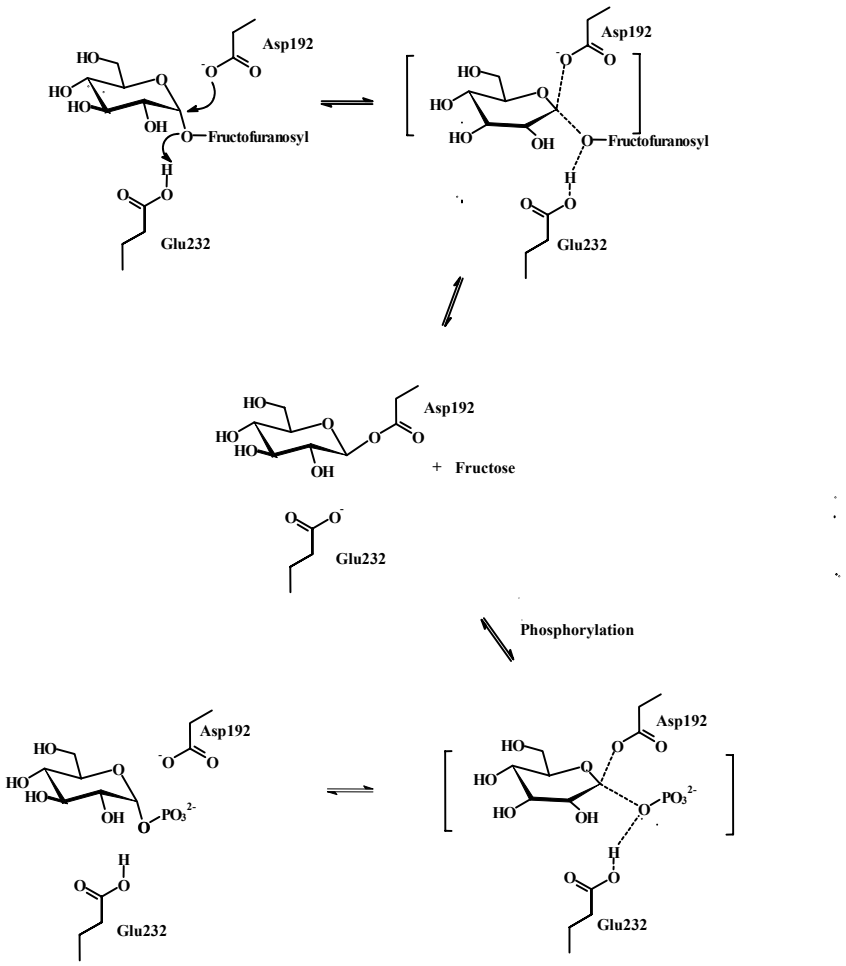


Figure 1: The sucrose phosphorylase reaction mechanism.

The reaction mechanism that is believed to operate in GH family 13 is a double-displacement reaction (Koshland 1953) involving a covalent glucose–enzyme intermediate. For sucrose phosphorylase from *P. saccharophila*, the existence of the intermediate was described in 1947 by Doudoroff et al. (1947). A mechanistic scheme accommodating the synthesis of α-D-glucose 1-phosphate from sucrose and inorganic phosphate is shown in Figure 1. The reaction is initiated by simultaneous protonation of the sucrose glycosidic bond by the proton donor (identified as Glu232) and a nucleophilic attack by Asp192 on the anomeric carbon of the glucose moiety. This leads to the covalently linked substrate–enzyme intermediate and the release of fructose. The intermediate can then react with phosphate (HPO_4^{2-} or $H_2PO_4^-$), and finally glucose 1-phosphate is released. Reaction with other nucleophiles such as water or saccharides is also possible. A detailed

kinetic study of SucP has not been performed yet, but sucrose phosphorylase from *P. saccharophila* has been thoroughly examined previously (Silverstein et al. 1967). For this enzyme, the initial rate of sucrose consumption was 56 times higher for phosphate than for water. It was also found that glucose, fructose, and phosphate could inhibit the enzyme and that the affinity for glucose was about 500 times greater than that for fructose or phosphate.

In this paper, we present the crystal structure of SucP (BiSP) refined at a resolution of 1.77 Å. It represents the first crystal structure of a sucrose phosphorylase and is the first structure of a phosphate-dependent enzyme from the GH family 13. This detailed structural investigation of a sucrose phosphorylase has increased our understanding of the basis of substrate specificity and will be an important tool for further engineering of enzymes involved in the starch and sucrose metabolism.

MATERIALS AND METHODS

Crystallization and data collection. Recombinant sucrose phosphorylase from *B. adolescentis* DSM20083 was expressed in *Escherichia coli* and purified from the cell-free extract as described in chapter 4 (Van den Broek et al. 2004). Crystallization conditions were screened according to the sparse-matrix method (Jancarik and Kim 1991) using commercially available buffers (Hampton Research) and the hanging-drop vapor-diffusion technique (Mcpherson 1992). Hanging drops were prepared by mixing 2.5 µl of protein solution (0.5–1.0 mg ml^{-1}, 10 mM Tris/HCl, pH 7.1) with 2.5 µl of precipitant solution (27 (w/v) % polyethylene glycol 4000, 0.1 M Tris/HCl, pH 8.5, 0.1 M Na-acetate) and equilibrated against 500 µl precipitant at 25 °C. Crystals grew within 3–14 days. All crystals were flash-cooled in liquid nitrogen prior to data collection. A native data set was collected to 1.8 Å resolution at 120 K on an in-house MAR345 image plate detector using Cu Kα radiation. A second native data set to 1.58 Å was collected at PSF, BESSY, Berlin, Germany, on a MARCCD.

For phase determination, a $Hg(CH_3CO_2)_2$ derivative was prepared. $Hg(CH_3CO_2)_2$ was dissolved in 1 ml precipitant and 0.3 µl was added to the hanging drops with crystals. Crystals were soaked for 8 days and brought to beamline 711, MAXLABII Synchrotron Facility, Lund, Sweden. A 2.05 Å SAD data set was collected at 110 K on a MARCCD.

Data from MAXLABII and in-house were processed with Denzo and Scalepack (Otwinowski and Minor 1997), whereas native data from BESSY were processed with Mosflm

(Leslie 1992) and Scala (Bailey 1994). All crystals belong to the orthorhombic space group $P2_12_12_1$, with two molecules in the asymmetric unit and with approximately the same cell dimensions: a = 55.04, b = 123.35, c = 151.48 Å (from high-resolution BESSY data).

TABLE 1: Crystallographic data and model refinement

Data collection and analysis			
	Native (in house)	$Hg(CH_3CO_2)_2$	Native (Bessy)
Resolution range, Å (outer shell)	20.0-2.00 (2.07-2.00)	51.9-2.04 (2.15-2.04)	23.3-1.58 (1.66-1.58)
Wavelength, Å	1.542	0.996	0.918
Completeness, % (outer shell)	87.9 (88.6)	99.4 (99.4)	97.8 (97.8)
Redundancy (outer shell)	3.4 (3.0)	9.2 (8.6)	3.7 (3.2)
R^a_{sym}, % (outer shell)	12.9 (32.9)	8.24 (23.5)	10.9 (53)
I/σI (outer shell)	9.1 (3.3)	8.2 (3.2)	4.3 (0.9)
Heavy atom refinement			
Number of Hg-sites		6	
Resolution range, Å		51.9-2.04	51.9-3.41
Phasing power[b], isomorphous acentric/centric		0.64/0.53	0.9/0.7
Phasing power[b], anomalous		0.61	1.50
R^c_{cullis}, isomorphous acentric/centric		0.84/0.83	0.82/0.80
R^c_{cullis}, anomalous		0.93	0.71
FOM[d] after SHARP acentric/centric		0.25/0.20	0.52/0.32
FOM[d] after Solomon		0.84	

Refinement statistics					
No. of Reflections (free)	Resolution, Å	R^e_{conv}/R^f_{ree}, %	No. of non-H protein/water/Tris atoms	R.m.s.d. bond, Å	R.m.s.d. angles, °
101,196 (5198)	20.0-1.77	15.8/19.1	7,912/1,412/16	0.006	1.4

[a] $R_{sym} = \sum_{hkl}(\sum_i(|I_{hkl,\,i} - \langle I_{hkl}\rangle|)) / \sum_{hkl,\,i}\langle I_{hkl}\rangle$, where $I_{hkl,\,i}$ is the intensity of an individual measurement of the reflection with Miller indicies h, k and l, and $\langle I_{hkl}\rangle$ is the mean intensity of that reflection.

[b] $R_{cullis} = \sum_{hkl}||F_{PH,\,hkl} \pm F_{P,\,hkl}| - F_{H,\,calc,\,hkl}| / \sum_{hkl}|F_{PH,\,hkl} - F_{P,\,hkl}|$, where F_{PH} is the structure factor of the heavy atom derivative, F_P is the structure factor of the native protein and $F_{H,\,calc}$ is the calculated structure factor for the heavy atom.

[c] Phasing power = $\sum_{hkl}F_{H,\,hkl} / \sum_{hkl}|F_{PH,\,obs,\,hkl} - F_{PH,\,calc,\,hkl}|$.

[d] Figure of merit.

[e] $R_{conv} = \sum_{hkl}(||F_{obs,\,hkl}| - |F_{calc,\,hkl}||) / |F_{obs,\,hkl}|$, where $|F_{obs,\,hkl}|$ and $|F_{calc,\,hkl}|$ are the observed and calculated structure factor amplitudes.

[f] R_{free} is equivalent to the R_{conv}, but calculated with 5% of the reflections omitted from the refinement process.

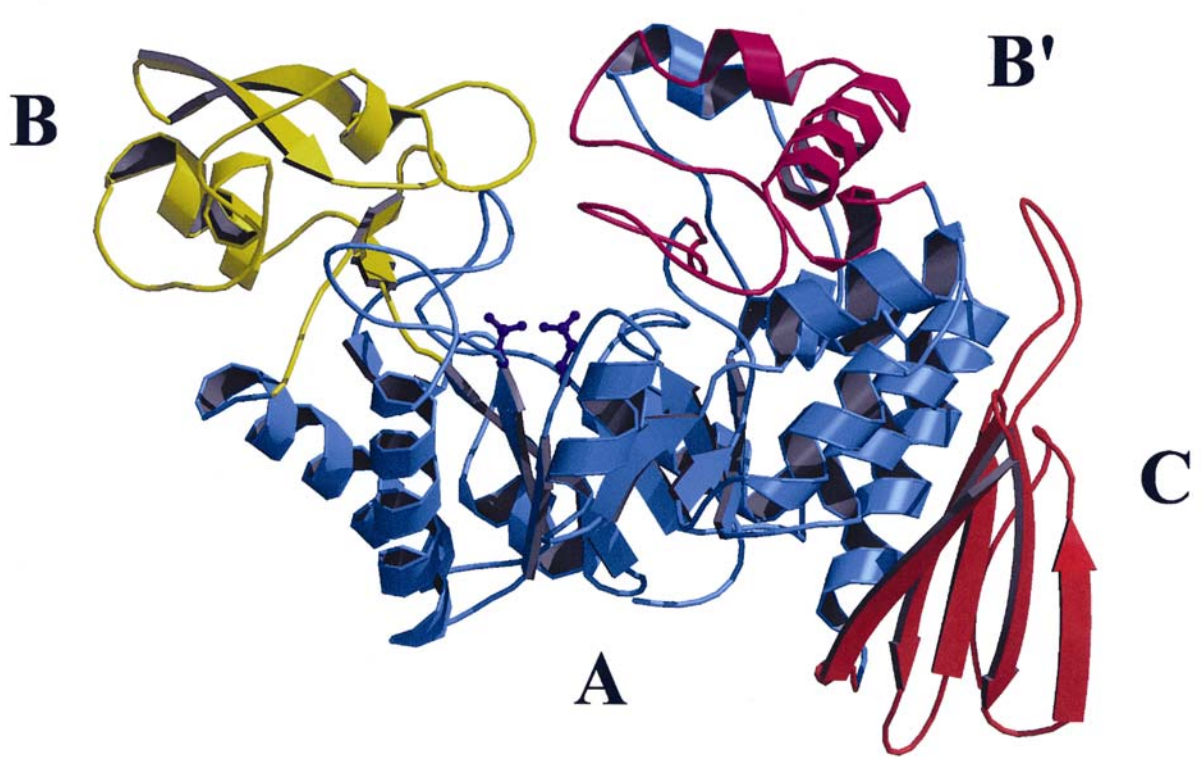


Figure 2: Richardson representation of the monomer structure of SucP (molecule A). Domain A is displayed in blue, domain B in yellow, domain B´ in magenta, and domain C in red. The catalytic active residues (Asp192 and Glu232) are shown in stick representation (purple).

Structure determination and refinement. SIRAS phases were calculated on the basis of the heavy atom derivative $Hg(CH_3CO_2)_2$. The heavy atom positions were identified from peaks in the anomalous and isomorphous difference Patterson maps using the CCP4 program suite (Bailey 1994). Heavy atom positions were refined and the phases calculated with the program Sharp (de La Fortelle and Bricogne 1997), followed by solvent density modification with a solvent content of 45.2% by the CCP4 program Solomon (Abrahams and Leslie 1996). The resulting electron density map was easily traced using the program Arp/Warp (Perrakis et al. 1999) with data to 1.58 Å resolution although $I/\sigma I$ was quite low below 1.77 Å. The model was refined with CNS (Brunger et al. 1998) with the mlf target function against data from 20.0 to 1.77 Å using a bulk solvent model and anisotropic *B*-factor correction. Refinement steps were accepted if they produced a lowering of R_{free}. Water molecules were picked among spherical peaks of 1.2σ in the $2Fo - Fc$ maps and were analyzed for hydrogen-bonding interaction with the protein or other water molecules. The

temperature factors were refined for every atom, but restrained to the temperature factors of neighboring atoms. The data and refinement statistics are listed in Table 1. Two SucP molecules (A and B) resulting in a total of 1,008 amino acid residues, two TRIS ions and 1,412 water molecules were included in the final model. Sixteen of the side chains were fitted with two conformations, and for 17 surface side chains some of the outermost atoms displayed high *B*-factors (> 40 $Å^2$).

The Ramachandran plot as calculated by the program PROCHECK (Laskowski et al. 1993) shows 89.9 % of the residues in the most favorable regions, 9.6 % in the additional allowed regions, 0.6 % (Phe156 and Asp446 in both molecules and Asp447 in molecule B) in the generously allowed regions and no residues in disallowed regions of the plot. The atomic coordinates as well as structure factors of SucP (BiSP) have been deposited in the Protein Data Bank (PDB) with the accession code 1R7A.

RESULTS AND DISCUSSION

Fold description. Two SucP molecules (A and B) are found in the asymmetric unit of the crystal. The fold is identical for both polypeptide chains and the structures can be superimposed with an rms deviation of 0.25 Å on 504 Cα atoms. The single polypeptide chain is folded into a structure with four domains, named A, B, B´, and C (Figure 2).

Domain A (residues 1–85, 167–291, and 356-435) is made up of eight alternating parallel β-strands (e1–e8) and α-helices (h1–h8), giving a $(\beta/\alpha)_8$-barrel common to the GH family 13. A characteristic of the $(\beta/\alpha)_8$-barrel enzymes is that the loops connecting strands to helices are much longer on average than those connecting helices to strands. SucP has two long loops (81 amino acid residues in the loop between e3 and h3, and 64 amino acid residues in the loop between e7 and h7) displaying structural elements and we have classified them as separate domains B and B´, respectively. Domain B (residues 86–166) contains two short antiparallel β-sheets and two short α-helices. The inner sheet (relative to the barrel) is formed by two strands (residues 88–90 and 160–162), and the outer sheet is formed by two strands (residues 140–145 and 148–153), flanked by the two α-helices (residues 94–102 and 125–129). Domain B´ (residues 292–355) is mainly a coil region, but contains one long α-helix (residues 312–325) and a short α-helix (residues 330–337).

Although the complete SucP sequence contains two cysteine residues, no disulfide bridges are found in the structure. One of the cysteines (Cys356) is exposed on the surface and is in this structure (with data generated by synchrotron radiation) oxidized to a sulfone, making it a modified amino acid (Csw356). However, in the structure generated by our in-house source it is still a cysteine. The other cysteine (Cys205) is located in the interior of the enzyme.

The 56 first residues of the C-terminal domain (Figure 2) form a single five-stranded anti-parallel β-sheet with a topology described as 1,1,1,1 in algebraic notation. After a turn, the last six residues are below this sheet and are found in an extended conformation. Strands 1–3 are connected by short hairpin loops, whereas the loop between strands 3 and 4 is formed by 16 residues. The tip of the loop is in proximity of the B′ domain and actually blocks some of the regions equivalent to oligosaccharide binding site OB2 in the amylosucrase structure (Skov et al. 2002). This probably makes the domain partially responsible for the change in substrate specificity between the two closely related enzymes.

It is remarkable that the fold of the C domain is not observed in any of the other known hydrolases in GH family 13. The fold of the β-sandwich domain observed in many of the family 13 hydrolases can, however, be derived from the SucP fold. Loop 3 in e.g. AS is even longer than in SucP and forms the second sheet of the β-sandwich. The sandwich domains terminate after a strand that coincides with SucP strand 4. These relations reflect the common origin of the hydrolases of GH family 13. A DALI search (Holm and Sander 1995) with the C domain alone revealed that the FhuA receptor (PDB accession no. 1BY5; Locher et al. 1998) displays the highest similarity (Z score 5.1). However, the aligned regions were only parts of the very long strands forming the transmembrane pore of FhuA. A corresponding region of the sucrose specific porin (1A0T; Forst et al. 1998) was also found to be similar (Z score 5.1) to the C domain. This suggests that the domain has a very unusual fold, and also that this domain conformation is highly dependent on interactions with the $(\beta/\alpha)_8$ barrel.

Dimer formation. Native molecular mass determination indicated that SucP is a homodimer in solution estimated by gel permeation experiments at pH 6.5 as described in chapter 4 (Van den Broek et al. 2004). To verify the dimer formation at crystallization pH, dynamic-light scattering was performed. Results confirmed dimer formation at crystallization pH 8.5 and at pH 6.5 (data not shown).

The symmetry of the crystal gave a number of possible dimers, which were submitted to the Protein–Protein Interaction Server (Jones and Thornton 1996). This analysis showed that two likely

dimers could be identified. Dimer 1 had a dimer interface area of 960 Å^2 (per monomer) while the area in dimer 2 was 910 Å^2. The total surface area of a monomer is 21,400 Å^2. Since both dimer interfaces are of significant size, direct hydrogen bonds and water-mediated hydrogen bonds were analyzed using PyMOL (DeLano Scientific LLC) and the CCP4 program Contact. The dimer 1 interface contains 11 hydrogen bonds, two salt bridges and five bridging water molecules. The dimer 2 interface is built of four hydrogen bonds, no salt bridges and eight bridging water molecules out of a large number of other water molecules located at the interface. The intermolecular forces in dimer 1 appear much stronger than those in dimer 2. In addition, β-sheet-type backbone interactions are formed across dimer 1. Therefore, we conclude that dimer 1 is the most likely solution dimer.

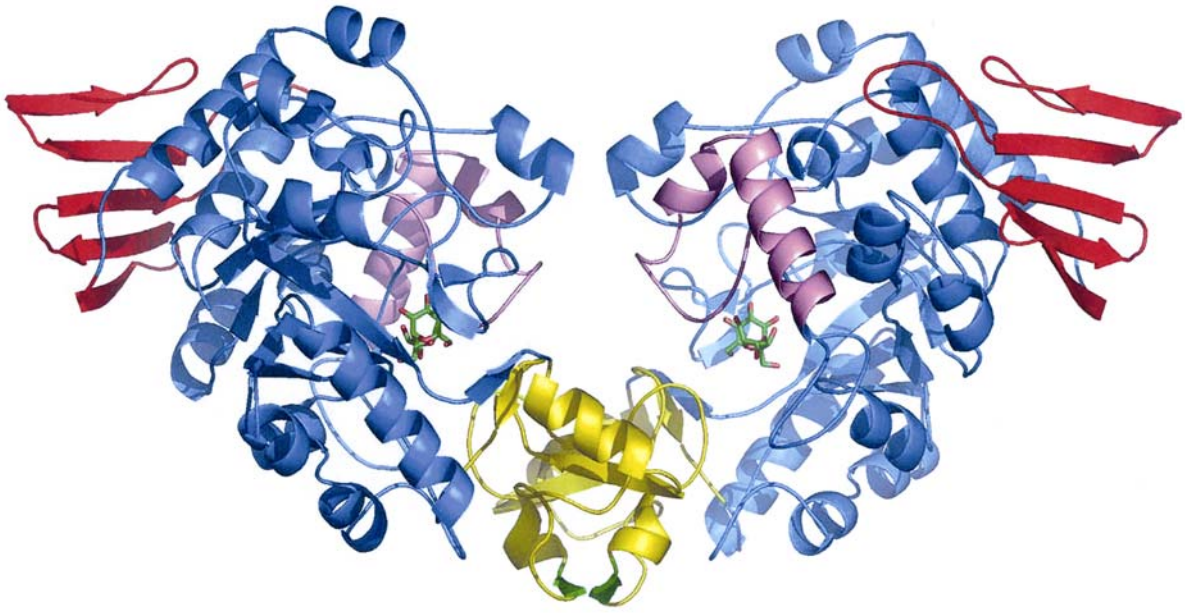


Figure 3: Richardson representation of the homodimer (molecule A and B) of SucP. The non-crystallographic two-fold symmetry axis is approximately vertical. The color coding is identical to Figure 2. The majority of the interactions are confined to the two B domains shown in yellow and the β-sheet type backbone interactions formed between the two domains are colored green. Sucrose molecules have been modelled into the active sites based on superposition of the structures of SucP and an AS:sucrose complex.

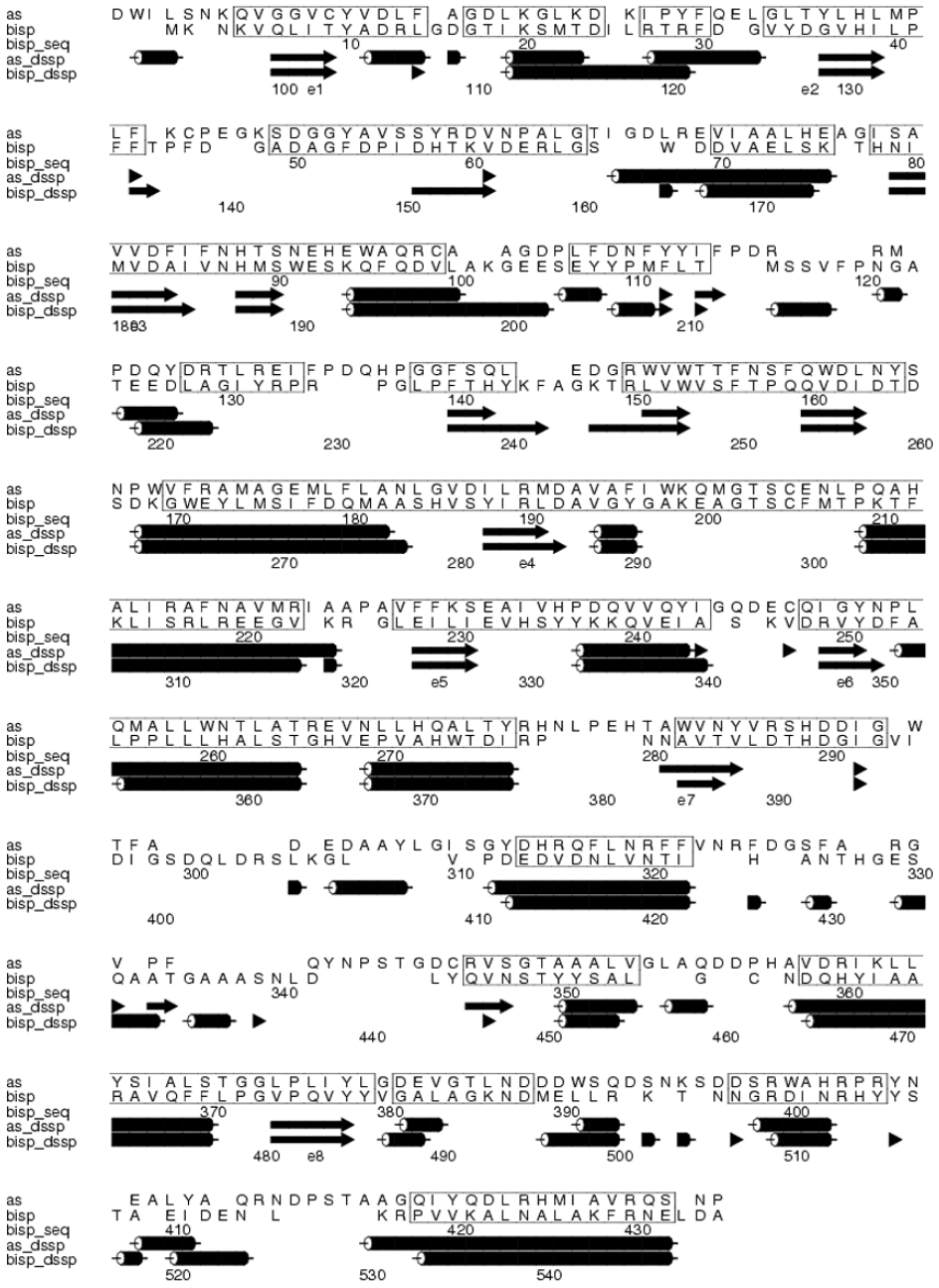


Figure 4: Structural alignment (Barton 1993; Russel and Barton 1992) of SucP and the structurally related enzyme amylosucrase (AS) using the Kabsch-Sander algorithm (Kabsch and Sander 1983).

The suggested SucP dimer is depicted in Figure 3. The majority of the interactions are confined to the two B domains, but interactions between the loop 8 regions of the two barrels are also observed. This results in a large cavity in the dimer, which includes the entrance to the two active sites. Other known structures of dimers in the GH family 13 are formed by the enzymes cyclomaltodextrinase (Lee et al. 2002), neopullulanase (Hondoh et al. 2003), and maltogenic amylase (Kim et al. 1999). In contrast to the SucP dimer, the other dimers were primarily formed by the hydrolase N-terminal domains. To our knowledge, the SucP structure represents the first assignment of a functional role of a B domain for dimerization in the entire GH family 13.

Active site. In a BLAST search with known 3D structure the highest alignment score for SucP was obtained with AS (De Montalk et al. 1999). The aligned regions cover the first half of the $(\beta/\alpha)_8$-barrel of AS (Figure 4). A pairwise alignment of the two sequences using the Clustal W algorithm (Thompson et al. 1994) also aligned the sequences after the unique amylosucrase N-terminal domain. The alignment covered the whole AS catalytic and C-terminal domain. Furthermore, the Pfam alignment server (Bateman et al. 1999) identifies a full amylase domain ($(\beta/\alpha)_8$-barrel and C-terminal sandwich domain) in SucP. This alignment shows that a B´ domain is present, as is the case for AS (Skov et al. 2001). The B´ domain is formed by loop 7 of the $(\beta/\alpha)_8$-barrel and comprises 64 residues as compared to 55 in AS. The loop is exceptional long in SucP and AS compared to the hydrolases of GH family 13 in general and probably explains the high BLAST alignment score against AS. In AS, this loop contains two oligosaccharide binding sites referred to as OB1 which includes the binding sites –1 to +5 of the hydrolases in GH family 13, and the high-affinity binding site OB2 somewhat distant from the active site (Skov et al. 2002). Hence, it has been suggested that this domain is largely responsible for the polymerase properties.

A DALI search (Holm and Sander 1995) with the whole SucP molecule clearly revealed AS as the protein with the highest similarity score, demonstrating that structural alignment of SucP and AS: substrate complexes is well suited as a basis for understanding the product profile and substrate specificity observed of SucP. The SucP active site was easily identified by superimposing the structures of SucP and AS. The superimposed active site region of SucP and of the AS:sucrose complex is shown in Figure 5A. The active site of AS has previously been compared to active sites of the hydrolases of family 13, and a high degree of similarity has been established (Skov et al. 2001). Thus, the AS active site can be regarded as representative for the entire family 13. The nucleophilic aspartates and the general acid/base glutamates (glutamine in the inactivated AS:sucrose complex) are seen to superimpose very well. Asp192 and Glu232 are found at the tips

of β-sheets 4 and 5 in the $(\beta/\alpha)_8$-barrel of the enzyme, as required for family 13 members. The distance between Asp192 Cα and Glu232 Cα is 5.5 Å in accordance with SucP being an α-retaining enzyme (Davies and Henrissat 1995). The third carboxylate conserved in the active site of family 13 enzymes is also found here (Asp290) and can be seen in Figure 5A. A Tris molecule is bound at the active site of SucP (Figure 5B) forming a short hydrogen bond from an oxygen atom to Oδ2 of Asp192 (2.6 Å) and in addition several hydrogen bonds to surrounding amino acid side chains (Asp50, His88, Glu232, and Arg399). In Figure 5B, it can be seen that Tris occupies several of the sucrose binding positions (based on a superimposition between SucP and the AS:sucrose complex) and this probably explains why it has previously been found in the active site of a number of structures of α-amylases (Aghajari et al. 1998).

The residues involved in the binding of the glucosyl moiety (in amylase nomenclature corresponding to subsite –1 interactions (Davies et al. 1997)) are structurally conserved. The SucP active site can accommodate the sucrose molecule without a need for major structural rearrangements, even though the fructosyl moiety surroundings are much less conserved as compared to the glucosyl binding site. Here, only Tyr196 is found in a position that is occupied by an aromatic residue in AS. No calcium ions were found in the structure, as probably expected of a phosphate-dependent enzyme. In SucP, a lysine Nζ (Lys199) is positioned at the site normally occupying the calcium ion in other family 13 enzymes. Several features of this calcium site are structurally conserved, as also found in AS (Skov et al. 2001) and oligo-1,6-glucosidase (Watanabe et al. 1997).

After the formation of the covalent intermediate, a phosphate ion must enter the active site (Figure 1). For steric reasons, the phosphate ion must bind at the position previously occupied by the fructosyl moiety, or at least very close to this position. Thus, one would expect a relatively positive electrostatic potential in this region. Several remarkable changes are observed between SucP and AS. At the position of AS Ile330 a histidine residue (His234) was found in SucP. This change introduces a positive charge in the active site, at least at acidic pH. In any case, the histidine residue is also positioned ideally for hydrogen bond formation to the phosphate ion and we expect a phosphate ion to bind here. This is also supported by the presence of Gln345 at an AS aspartate position. Another change is perhaps more surprising. In SucP, Asp342 now occupies an AS arginine position and probably contribute to a less positive electrostatic potential. These latter findings could indicate that structural rearrangements are involved at some stage of the reaction mechanism.

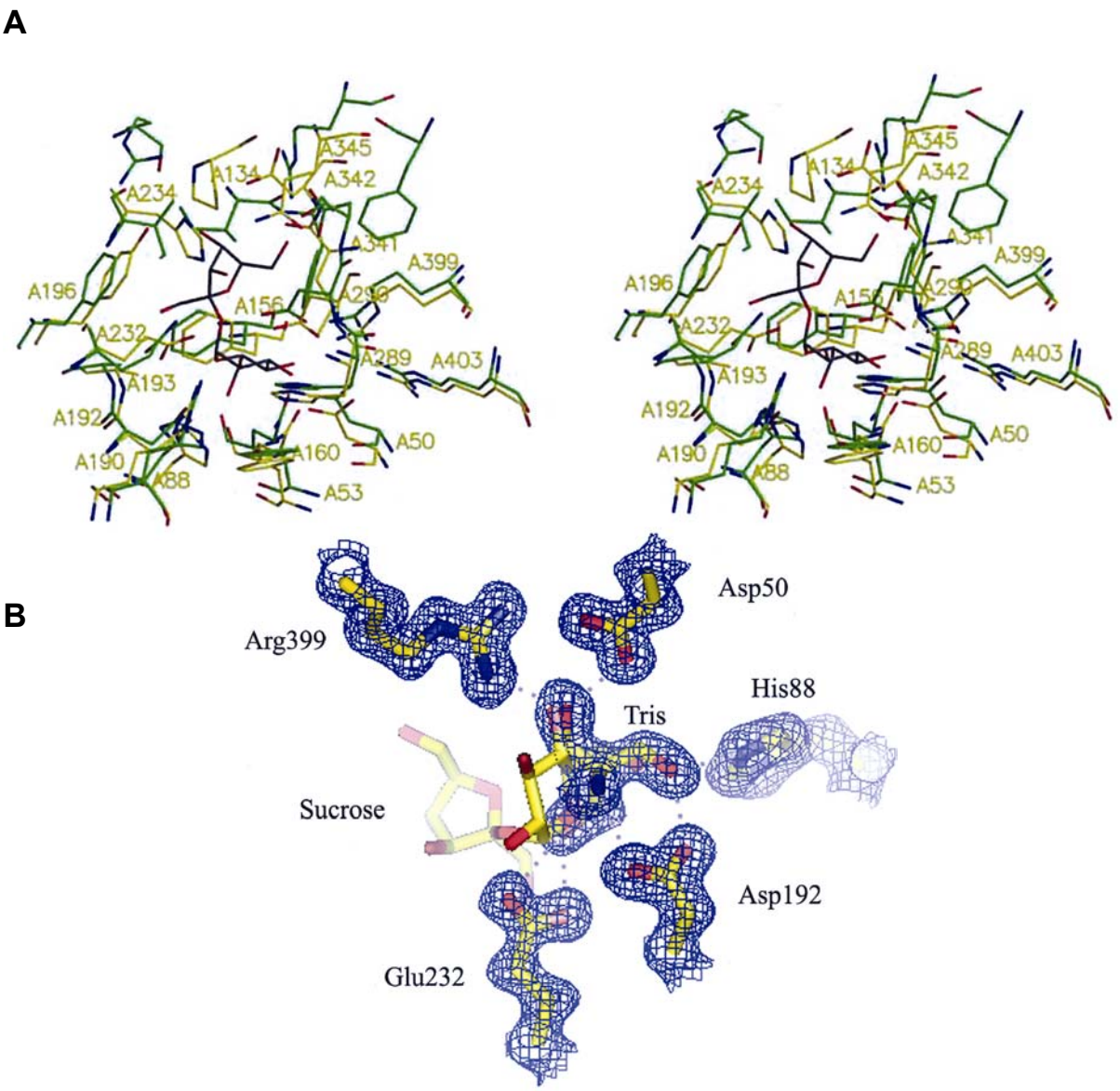
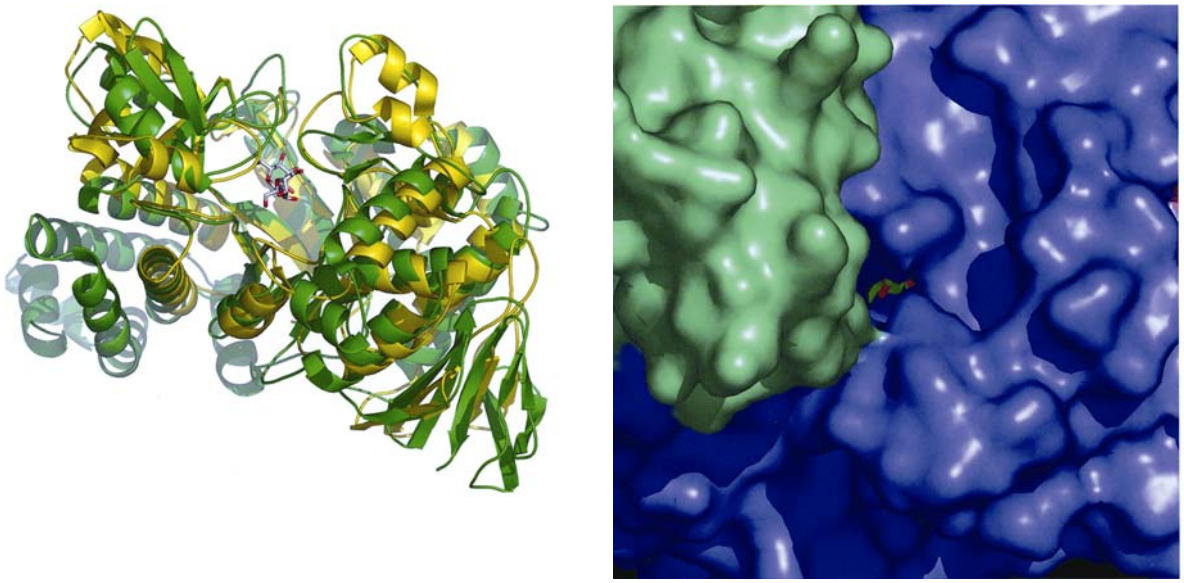


Figure 5: The active site of SucP. (**A**) Superposition of the active site residues of SucP (in green) and amylosucrase (AS; in yellow). The sucrose molecule of the AS:sucrose complex is shown in black. Heteroatoms are in standard colours: nitrogen atoms are blue and oxygen atoms red. (**B**) Zoom on the Tris molecule observed in the active site of SucP. The final 2Fo–Fc electron density contoured at 1σ is shown in blue. Hydrogen bonds are displayed as dotted line

Substrate access channel. In Figure 6A, the structures of SucP and AS are viewed down the active site access channel, with the sucrose molecule of the AS structure included (Mirza et al. 2001). The SucP channel appears much smaller than that of AS. A loop from the B´ domain seems to be largely

responsible for reduced size and this observation again stresses the importance of the B´ domain and its role in modulating the function of family 13 enzymes. In SucP, the topology of the B´ domain disfavors oligosaccharide binding and reduces the size of the substrate access channel, whereas in the polymerase AS the B´ domain topology is essential for oligosaccharide binding. Hydrolases such as amylases have no B´ domain and this results in a cleft-like active site topology. As shown in Figure 6B, the SucP dimer formation does not block the access to the active sites of the dimer.

SucP catalyzes a reaction with a smaller secondary substrate than AS, and can consequently function with a smaller active site access channel. However, one basic problem is the same, namely the protection of the covalent intermediate against hydrolysis. AS probably solves this problem by having oligosaccharides block the substrate access channel after fructose release (Skov et al. 2002). How this problem is solved in SucP will have to await structure determinations of intermediates in the reaction cycle.



A **B**

Figure 6: The substrate access channel. (A) An aligned cartoon representation of SucP (in green) and amylosucrase (in yellow) viewed down the substrate access channel, with the sucrose molecule of AS included in stick representation. (B) PyMOL picture of a close-up of the solvent accessible surface representation of the SucP homodimer (molecule A and B) including the sucrose molecule from the AS:sucrose complex. The surfaces of molecule A and B are colored in blue and green, respectively.

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Chapter 6

Cloning and characterization of arabinoxylan arabinofuranohydrolase-D3 (AXHd3) from *Bifidobacterium adolescentis* DSM20083

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ABSTRACT

Arabinoxylan arabinofuranohydrolase-D3 (AXHd3) from *Bifidobacterium adolescentis* releases only C3-linked arabinose residues from double-substituted xylose residues. A genomic library of *B. adolescentis* DSM20083 was screened for the presence of the *axhD3* gene. Two plasmids were identified containing part of the *axhD3* gene. The nucleotide sequences were combined and three open reading frames (ORFs) were found. The first ORF showed high homology with xylanases belonging to family 8 of the glycoside hydrolases and this gene was designated *xylA*. The second ORF was the *axhD3* gene belonging to glycoside hydrolase family 43. The third (partial) ORF coded for a putative carboxylesterase.

The *axhD3* gene was cloned and expressed in *Escherichia coli*. Several substrates were employed in the biochemical characterization of recombinant AXHd3. The enzyme showed the highest activity toward wheat arabinoxylan oligosaccharides. In addition, β-xylanase from *Trichoderma* sp. was able to degrade soluble wheat arabinoxylan polymer to a higher extent, after pretreatment with recombinant AXHd3. Arabinoxylan oligosaccharides incubated with a combination of recombinant AXHd3 and an α-L-arabinofuranosidase from *Aspergillus niger* did not result in a higher maximal release of arabinose than incubation with these enzymes separately.

INTRODUCTION

Bifidobacteria can have several health-promoting effects in humans (Gibson 1998; Gibson and Roberfroid 1995). To increase the amount of bifidobacteria in the gastrointestinal tract, they can be added to the diet, which is an example of a probiotic. Another way to increase the amount of bifidobacteria is through the consumption of a prebiotic. The definition of a prebiotic is 'a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that have the potential to improve host health' (Gibson and Roberfroid 1995). Examples of prebiotic compounds are fructo-oligosaccharides (FOS), α-galacto-oligosaccharides (GOS), β-galacto-oligosaccharides (TOS), and xylo-oligosaccharides (XOS; Crittenden and Playne 1996). An in vivo study with XOS intake by humans showed the growth promotion of bifidobacteria (Okazaki et al. 1990).

The Japanese market started to pioneer the addition of XOS in food as a prebiotic and the first synbiotic, a combination of a probiotic and prebiotic, containing bifidobacteria and XOS and some other components, was marketed in 1993 (Crittenden and Playne 1996). Nowadays, a whole range of products containing XOS is available (Vásquez et al. 2000). It is known that xylan is hardly fermented by bifidobacteria (Crociani et al. 1994), whereas XOS and/or arabinoxylan oligosaccharides (AXOS) can be easily fermented (Campbell 1997; Crittenden et al. 2002; Jaskari et al. 1998; Okazaki et al. 1990; Van Laere et al. 2000). However, relatively little is known about XOS/AXOS-degrading enzymes from bifidobacteria. Only a β-D-xylosidase purified from *B. breve* K-100 was shown to be able to release xylose from xylan (Shin et al. 2003a). The genome sequence of *B. longum* NC2705 revealed that most of the sugar-modifying enzymes were related to arabinan and xylan degradation. A total of 14 putative arabinoxylan degrading-enzymes were identified (Schell et al. 2002). So far, only an α-L-arabinofuranosidase from *B. longum* B667 has been cloned and characterized (Margolles and De Los Reyes-Gavilán 2003). This enzyme showed exo-activity and could only release 25% of the arabinose residues from flour arabinoxylan upon prolonged incubation. Van Laere et al. (1997; 1999) isolated two different arabinofuranosidases from *B. adolescentis* able to release arabinose residues from arabinoxylan. These enzymes were named arabinofuranohydrolase-D3 (AXHd3; which hydrolyzed only C3-linked arabinose residues from double-substituted xylose residues) and AXHm23 (which released only arabinose residues that were C2 or C3 linked to a singly-substituted xylose residue). These two enzymes, together with a β-xylosidase were able to degrade AXOS completely (Van Laere et al. 1999).

Here, we report the identification of the *axhD3* gene and a xylanases gene (*xylA*) from *B. adolescentis* and the characterization of recombinant AXHd3. Also, their role in AXOS degradation is discussed.

MATERIALS AND METHODS

Enzyme purification and internal amino acid sequencing. The AXHd3 from *B. adolescentis* was purified as previously described (Van Laere et al. 1997; 1999). The protein was concentrated with a 10 kDa filter (Amicon) and the N-terminal sequence and internal amino acid sequences of the enzyme were determined (Eurosequence, Groningen, The Netherlands). The gel electrophoresis and blotting of the concentrated sample was as described in chapter 3 (Van den Broek et al. 2003).

Genomic library and screening. A PCR reaction was performed with primers (Table 1) designed from obtained internal amino acid sequences (Figure 1), Supertaq DNA polymerase (Sphearo Q), and the genomic DNA of *B. adolescentis*, which was isolated as previously described in chapter 2 (Van den Broek et al. 1999). The reaction conditions were: 94 ^{o}C for 5 min, followed by 30 cycles of 94 ^{o}C for 30 s, 50 ^{o}C for 30 s, and 72 ^{o}C for 2 min, ending with 72 ^{o}C for 10 min. PCR products obtained with the primers 1BFOR/2REV and 1BFOR/4REV were isolated from an agarose gel using the MBI DNA extraction kit (Fermentas). The purified product was cloned into pGEM-T Easy (Promega) for cloning purposes and nucleotide sequencing.

Primers 1BFOR and 4REV were used in PCR for digoxigenin (DIG)-labeling the *axhD3* fragment for colony hybridization. The genomic library of *B. adolescentis* DSM20083 was constructed as described in chapter 2 (Van den Broek et al. 1999). Colony hybridization and detection was performed following the instruction of the manufacturer of the DIG-labeling kit (Boehringer Mannheim).

Sub-cloning of the *axhD3* gene. To facilitate the over-expression of recombinant AXHd3, the *axhD3* gene was sub-cloned from pGEM-T Easy into pET22b (Novogen). Using pGEM-T Easy plasmid DNA as the template, the gene sequence was amplified by PCR using KOD Hot Start DNA polymerase (Novogen), the primers AXHPETFOR and AXHPETREV (Table 1), and a thermal program comprising 25 cycles of 94 ^{o}C for 1 min, 61 ^{o}C for 1 min, and 68 ^{o}C for 1 min 45 s, following five initial cycles with an annealing temperature of 45.1 ^{o}C. The 1,569 kb PCR product contained engineered *Nde*I and *Xho*I restriction endonuclease sites at the 5′ and 3′ termini,

respectively, and the original stop codon was deleted to place *axhD3* in frame with the C-terminal poly-histidine purification peptide of the pET22b expression vector. The undigested PCR product was purified from an agarose gel using a QIAquick gel extraction kit (Qiagen), ligated into pCR-Blunt (Invitrogen) and sub-cloned into pET22b on a *Nde*I/*Xho*I restriction fragment, generating pRML2.

TABLE 1: Oligonucleotide primers used in the PCR experiments

Primer	Sequence (5′→3′)[a]
AXHFOR	ATGATGATTACCTCAACTAATC
AXHREV	TCATTGCTCTCTTTCCTTCG
AXHPETFOR	CATATGATGATTACCTCAACTAATC
AXHPETREV	CTCGAGTTGCTCTCTTTCCTTCG
1AFOR	TAYGAYCTBGTNCAYTGGGA
1AREV	TCCCARTGNACVAGRTCRTA
1BFOR	GTNCAYTGGGARTTYATHG
1BREV	CDATRAAYTCCCARTGNAC
2FOR	ATCGTSCARGAYGAYCCNCA
2REV	CYTGNGGRTCRTCYTGSACGA
4FOR	ATGGGYCCBGTSACNGTNG
4REV	GCSACNGTSACVGGNCCCA

[a] Nucleotide codes: N: A, T, C, or G; W: A or T; S: C or G; Y: C or T; V: A, C, or G; R: A or G; D: A, T, or G

Over-expression and purification of recombinant AXHd3. *Escherichia coli* BL21(DE3), transformed with pRML2 was cultured on 20 g LB agar l^{-1} supplemented with 50 mg ampicillin l^{-1} for 16 h at 37 ^{o}C. A single colony was used to inoculate 1 l LB liquid growth medium, supplemented with 50 mg ampicillin, that was incubated for 16 h at 37 ^{o}C with shaking at 140 rpm. The starter culture, 10 ml, was used to inoculate a 2 l Erlenmeyer flask containing 1 l LB liquid growth medium, supplemented with 50 mg ampicillin, that was incubated at 37 ^{o}C with shaking at 140 rpm. When the optical density at 600 nm (OD_{600}) reached 0.5, the temperature was reduced to 23 ^{o}C and over-expression of recombinant AXHd3 was induced by the supplementation of the

growth medium with 1 mM isopropyl-thio *β*-D-galactoside (IPTG). After incubation for a further 5 h, the bacterial cells were harvested by centrifugation and re-suspended in start buffer (10 mM HEPES buffer, pH 7.5, 10 mM imidazole, 500 mM NaCl). After sonication, cell debris was removed by centrifugation for 30 min at 28,000 *g* at 4 ^{o}C, and the supernatant was loaded onto a chelating Sepharose column (1.6 x 15 cm; Amhersham), charged with $NiSO_4$ and equilibrated with start buffer. Bound proteins, due to the presence of the C-terminal poly histidine tail, were washed with 100 ml of start buffer, followed by 100 ml of start buffer containing 50 mM imidazole. Recombinant AXHd3 was eluted with a linear gradient of imidazole from 50 to 500 mM. Purity was assessed by SDS-PAGE (Laemmli 1970) and fractions containing pure recombinant AXHd3 were pooled and precipitated by increasing the $(NH_4)_2SO_4$ concentration to 3.2 M. Precipitated recombinant AXHd3 was washed 3 times with 3.2 M $(NH_4)_2SO_4$.

Protein concentration was determined by the method of Bradford (1976) or the Folin/Lowry procedure (Lowry et al. 1951), using bovine serum albumin as the standard.

Enzyme assays. Substrates for α-L-arabinofuranosidase were *p*-nitrophenyl-α-L-arabinofuranoside (Sigma), medium viscosity wheat arabinoxylan (Megazyme), sugar-beet arabinan (Megazyme), β-xylanase degraded wheat arabinoxylan (approx. 5% hydrolyzed), and Arabinazyme and Xylazyme AX test tablets (Megazyme). *p*-Nitrophenyl-α-L-arabinofuranoside was prepared at a concentration of 5 mM in 100 mM sodium phosphate buffer (pH 6.0). Arabinoxylan, sugar-beet arabinan and depolymerized arabinoxylan were used at a concentration of 10 g l^{-1} in 100 mM sodium phosphate buffer (pH 6.0).

Arabinofuranosidase was assayed as follows. To pre-equilibrated wheat arabinoxylan, depolymerized wheat arabinoxylan, or sugar-beet arabinan (0.5 ml, 10 g l^{-1}) in 100 mM sodium phosphate buffer (pH 6.0), 0.2 ml recombinant AHXd3 was added and subsequently mixed and incubated at 40 ^{o}C. The reaction was terminated after 3, 6, 9, and 12 min by placing the tube in boiling water for 3 min. A zero time value was obtained by incubating the enzyme in boiling water for 3 min before adding the substrate. To each tube, 2.5 ml 100 mM Tris/HCl buffer (pH 8.6) containing 2 mM EDTA was added, followed by 0.1 ml NAD^+ (10 g l^{-1}). The absorbance was measured at 340 nm (A_1) and then 10 µl of galactose dehydrogenase (100 units ml^{-1}; Megazyme) were added. The absorbance was measured again (A_2), after incubation at ~ 25 ^{o}C for 40 min (or at 40 ^{o}C for 20 min). Free L-arabinose was calculated from Δ ($A_2 - A_1$) according to the instructions of the Lactose/Galactose kit (Megazyme).

pH activity was determined by incubating 0.2 ml recombinant AXHd3 with 0.5 ml depolymerized arabinoxylan (10 mg ml^{-1}) in 100 mM citrate/phosphate buffer at pH 4.0–7.0, otherwise as described above.

Action on *p*-nitrophenyl-α-L-arabinofuranoside was determined by incubating 0.2 ml suitably diluted enzyme with 0.2 ml of substrate (5 mM) in 100 mM sodium phosphate buffer (pH 6).

Assays with Arabinazyme and Xylazyme AX tablets was performed according to the manufacture (Megazyme).

Hydrolysis of arabinoxylan. Depolymerized wheat arabinoxylan (5 ml, 2 g l^{-1}) in either 100 mM sodium phosphate buffer (pH 6.0) or sodium acetate buffer (pH 4.5) was incubated with recombinant AXHd3 (7 units on wheat arabinoxylan, pH 6.0), *Aspergillus niger* arabinofuranosidase (500 units on *p*-nitrophenyl-α-L-arabinofuranoside, pH 4.5; Megazyme) or a mixture of both enzymes (pH 5.0) at 40 ^{o}C. Aliquots (0.5 ml) were removed at various time intervals and incubated at 100 ^{o}C to inactivate enzyme activity. Distilled water (1.5 ml) was added to each sample and aliquots (0.2 ml) were removed for the determination of released arabinose, using the galactose dehydrogenase/NAD^{+} assay. Total carbohydrate concentration was determined using the phenol-sulfuric acid procedure (Dubois et al. 1956) with an arabinose/xylose (40:60) standard solution. The degree of hydrolysis was calculated from the arabinose released, as a percentage of total carbohydrate.

Wheat arabinoxylan (1 l, 10 g l^{-1}) in 2 mM sodium phosphate buffer (pH 6.0) was incubated with 1,000 units recombinant AXHd3 at 40 ^{o}C. Aliquots (1 ml) were removed at various time-intervals for the determination of free arabinose levels (i.e. extent of hydrolysis). After 2 h, the solution was incubated at 100 ^{o}C for 20 min to inactivate recombinant AXHd3. To this solution 10 ml sodium acetate buffer (1 M, pH 4.5) were added and the pH was adjusted to 4.5. β-Xylanase M1 (2,000 units; *Trichoderma* sp.; Megazyme) was added and the solution was incubated at 40 ^{o}C for 60 min and then at 100 ^{o}C to inactivate enzyme activity. In a parallel experiment, wheat arabinoxylan (1 l, 10 g l^{-1}) in 10 mM sodium acetate buffer (pH 4.5) was incubated with 2,000 units β-xylanase M1 (*Trichoderma* sp.) at 40 ^{o}C for 60 min and then at 100 ^{o}C for 20 min. In both cases, the reaction solutions were concentrated to 100 ml, centrifuged at 12,000 *g* to remove insoluble protein material and 25 ml (approx 2.5 g) applied to a column of Bio-Gel P-2 (5 x 95 cm; BioRad) and chromatographed at 60 ^{o}C with elution by degassed distilled water. Fractions (20 ml) were collected and analyzed for carbohydrate using the phenol-sulfuric acid procedure.

Nucleotide sequence accession number. An automated DNA Sequencer 373 (Applied Biosystems) was used for determining the nucleotide sequence of the genes. The DNA sequence data (*axhD3*, *ces*, and *xylA*) were submitted to the GenBank nucleotide databases under the accession number AY233379. The BLAST2 program (Altschul et al. 1997) was used for searching sequence homologies.

RESULTS

Identification and cloning of the arabinofuranohydrolase-D3 gene and an endo-xylanase gene from *B. adolescentis*. The AXHd3 enzyme (Van Laere et al. 1997; 1999) was subjected to N-terminal and internal amino acid sequencing for cloning purposes. The N-terminus was blocked, but four internal amino acid sequences were obtained (Figure 1). From these sequences, different primers were designed (Table 1) and different combinations were used in PCR reactions for the specific amplification of parts of the *axhD3* gene from *B. adolescentis*. The combinations 1AFOR/2REV, 1BFOR/2REV, 1AFOR/4REV, and 1BFOR/4REV resulted, respectively, in fragments of approximately 400, 400, 1,200, and 1,200 bp, as estimated by agarose gel electrophoresis. Primers 1AFOR and 1BFOR were designed from the same internal fragment and therefore only the fragments obtained with the primer combinations 1BFOR/2REV and 1BFOR/4REV were sequenced. The combination 1BFOR/2REV resulted in a PCR fragment of 371 bp and 1BFOR/4REV gave a fragment of 1,284 bp. Primer combination 1BFOR/4REV was used for DIG-labeling of the PCR product which was used for colony hybridization. Two different types of plasmids hybridized with the labeled PCR fragment. The plasmids of these colonies were sequenced and both types of colony contained a part of the *axhD3* gene in their plasmid. The nucleotide sequence of both plasmids was combined and resulted in a DNA sequence of 3,674 bp. Two open reading frames (ORFs) and one partial ORF were identified. The first ORF was 1,140 bp, the second was 1,590 bp and the partial ORF contained 921 bp.

The deduced amino acid sequence of the first ORF contained 379 amino acids, encoding a protein of 43,785 Da. A database search revealed 52% identity with xylanase Y from *Bacillus halodurans* (NP_242971; AAN16076), 31% identity with an endoglucanase Y from *Cytophaga hutchinsonii* (ZP_00309326), 31% identity with and an endo-1,4-β-xylanase precursor from an uncultured bacterium (AAS85781), and 27% identity with xylanase Y from *Bacillus* sp. KK-1

(AAC27700). The gene was designated *xylA*. The xylanases are members of family 8 of the glycoside hydrolases (Henrissat 1991) and therefore the putative *xylA* was classified in this family.

001 MMITSTNPMV YTDFPDPDII RVGDVYYMAT TTMHFTPGCD ILR**SYDLVHW**

Fraction 1

051 **EFIAHALN**IV ADTPEERLEC EGANAYGRGM WAPSLRYHRG TWYVLFAAND

101 THTSYLLTAD DPCGPWRKRE LDGFYYDSGL FFDDDDRAYV VHGQSTLRIT

151 ELNPELSGPM PGGLDR**VIVQ DDPQADLGYE G**SHLYKHDGR YYVFTCHFPQ

Fraction 3

201 GKGKTEACLM AESLDGAFEV REIIEDDLSF HGYGVAQGGM VDTPDGDWYA

251 FMMQDRGGVG RVPILMPMRF GEDGFPVVGE NGKVPQSVSV PAASCAEPVT

301 PINGSEFIAR YNAEGGVDAN CLQPYWQFNH ISHNEYWSLA ERPGAFRLHS

351 GRISSNLNHA WNTLTQR**TMG PVTVAEVTVD A**STLHDGDFA GLAAFQGCYS

Fraction 2

401 YIALTRRNGR TMLTVQYKPA NDDSIFSDND WDSPAVTDAE IMADADCMRL

451 RAVYDFTDCK DEVTFFYR**DA DTPES**EWCPL GTAHRMIFKM DHFTGCRIGL

Fraction 4

501 FLYSTKETGG IADFYDFAYS TPNTKEREQ

Figure 1: Amino acid sequence of AXHd3 from *Bifidobacterium adolescentis*. Fractions obtained by internal amino acid sequencing are in *bold face type and underlined*.

The deduced amino acid sequence of the second ORF contained 529 amino acids, encoding a protein of 59,403 Da. The deduced amino acid sequence contained all four internal amino acid sequences of the native AXHd3 (Figure 1). Therefore, the gene was designated *axhD3*. The gene

revealed 38% and 34% identity with a β-xylosidase from *C. hutchinsonii* (ZP_00310780) and an endo-1,4-β-xylanases from *Caldocellum saccharolyticum* (AAB87371), respectively. For a possible β-xylosidase from *Clostridium acetobytylicum* (AAK76859), belonging to family 43 of the glycoside hydrolases, 32% identity was found. As *axhD3* showed homology with members of glycoside hydrolase family 43 it was classified in this family. Both glycoside hydrolase (GH) family 8 and 43 have an inverting catalytic mechanism.

The deduced amino acid sequence of the partial ORF contained 307 amino acids. A database research revealed 44% identity with a putative esterase from *Salmonella enterica* subsp. *enterica* serovar *Thypi Str*. CT18 (NP_455874) and a putative carboxylesterase from *S. typhimurium* LT2 (NP_460582). This gene was designated *Ces* and showed highest homology with members of carbohydrate esterase (CE) family 10. Most members of this family are esterases acting on non-carbohydrate substrates.

Overview of arabinoxylan degrading enzymes from *Bifidobacterium* spp. The amino acid sequence of *axhD3* was compared with the other known amino acid sequences of arabinoxylan-degrading enzymes from *B. longum*. A dendrogram was constructed using the Clustal method; and two distinct groups were observed, one of which represented mainly members of GH family 51, while the other group contained exclusively members of GH family 43 (Figure 2). The group containing the members of GH family 51 consisted of putative α-L-arabinosidases and α-L-arabinofuranosidases. The cloned α-L-arabinofuranosidase from *B. longum* B667 (AY259087; Margolles and De Los Reyes-Gavilán 2003) was similar to the α-L-arabinofuranosidase from *B. longum* NCC2705 (BL1166; Schell et al. 2002). The two putative related α-arabinofuranosidases II (BL0187; BL0184) belonging to GH family 43 (http://afmb.cnrs-mrs.fr/CAZY/index.html) were placed in the dendrogram in the group containing the members of GH family 51. AXHd3 showed the highest homology with a putative extracellular exo-xylanase (BL1544; *xynF*) from *B. longum* NCC2705.

Substrate specificity of recombinant AXHd3. A recombinant AXHd3 containing an additional histidine-tag to facilitate enzyme purification was expressed in *E. coli*. The molecular mass was approximately 60 kDa as estimated by SDS-PAGE and is in agreement with the deduced amino acid sequence of *axhd3*, taking into account the poly-histidine tag at the C-terminus (1,083 Da). The pH optimum of recombinant AXHd3 was pH 6.0, which was similar to native AXHd3 (Van Laere et al. 1999). The enzyme was characterized using various substrates and showed the highest activity toward oligosaccharides obtained from wheat flour arabinoxylan digested by endo-β-xylanase

(Table 2). Whereas native AXHd3 (Van Laere et al. 1999) apparently showed no detectable activity towards *p*-nitrophenyl-α-L-arabinofuranoside, recombinant AXHd3 was able to release *p*-nitrophenol from this substrate at a very low rate, as a large amount of the enzyme could now be employed. The recombinant AXHd3 showed no endo-activity against xylan and arabinan.

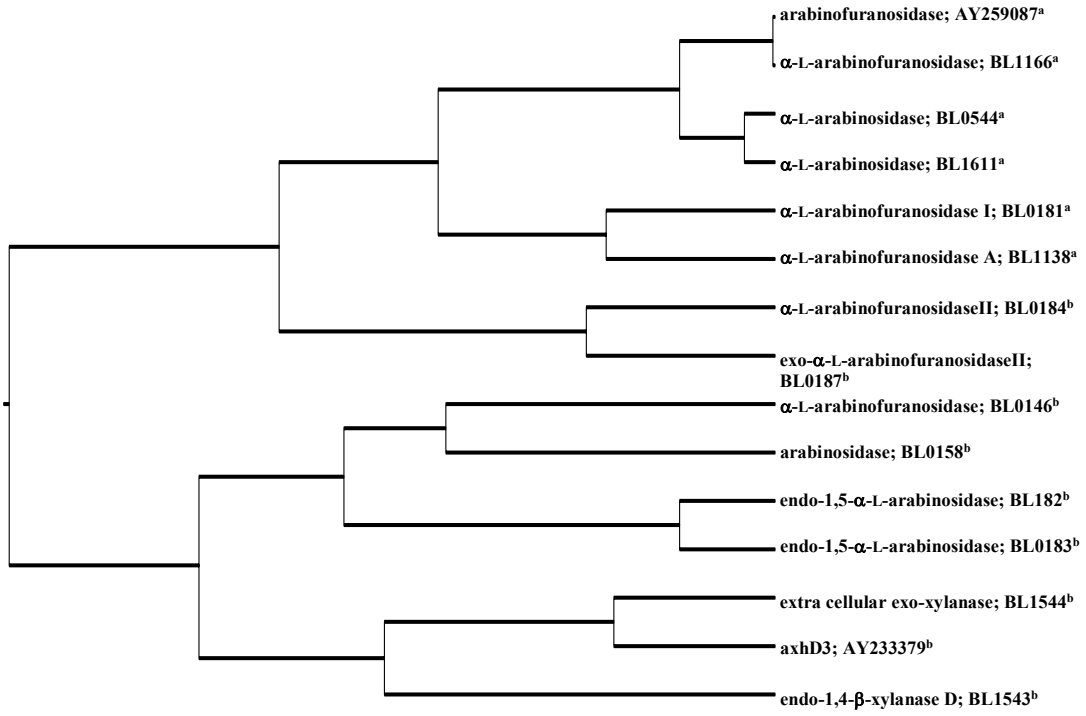

Figure 2: Dendrogram based on amino acid sequences of arabinoxylan-degrading enzymes from *Bifidobacterium* spp. The dendrogram was constructed using the Clustel method.
[a] Glycoside hydrolase family 51. [b] Glycoside hydrolase family 43.

TABLE 2: Activity of AXHd3 from *Bifidobacterium adolescentis* DSM20083 on various substrates

Substrate	Activity (units/mg protein)
Wheat flour arabinoxylan	28.3
Endo-β-xylanase treated wheat flour arabinoxylan (AXOS)	90.4
Sugar-beet arabinan	4.0
p-Nitrophenyl-α-L-arabinofuranoside	0.095
Arabinazyme Tablets (*endo*-1,5-α-L-arabinanase)	< 0.001
Xylazyme Tablets (*endo*-1,4-α-D-xylanase)	< 0.001

Soluble wheat arabinoxylan was incubated with an endo-β-xylanase from *Trichoderma* sp. (Figure 3A) or with recombinant AXHd3 followed by incubation with endo-β-xylanase (Figure 3B) to investigate any synergistic mechanism between the two enzymes. Size exclusion chromatography showed that wheat arabinoxylan was degraded to a higher extent by β-xylanase after pretreatment with recombinant AXHd3. The polymeric fraction was reduced and substantially higher amounts of mono-, tri-, tetra-, and hexamers were produced. The synergistic effect was also tested for the release of arabinose from wheat arabinoxylan preincubated with endo-β-xylanase by the recombinant AXHd3 and an α-L-arabinofuranosidase from *A. niger* (Figure 4). High amounts of α-L-arabinofuranosidase were used to obtain maximal release of arabinose. Recombinant AXHd3 released approximately 10% of the arabinose residues from AXOS and the α-L-arabinofuranosidase from *A. niger* was able to release approximately 40% of the arabinose residues. However, no increase for the amount of maximal release of arabinose was observed after incubation with both enzymes together.

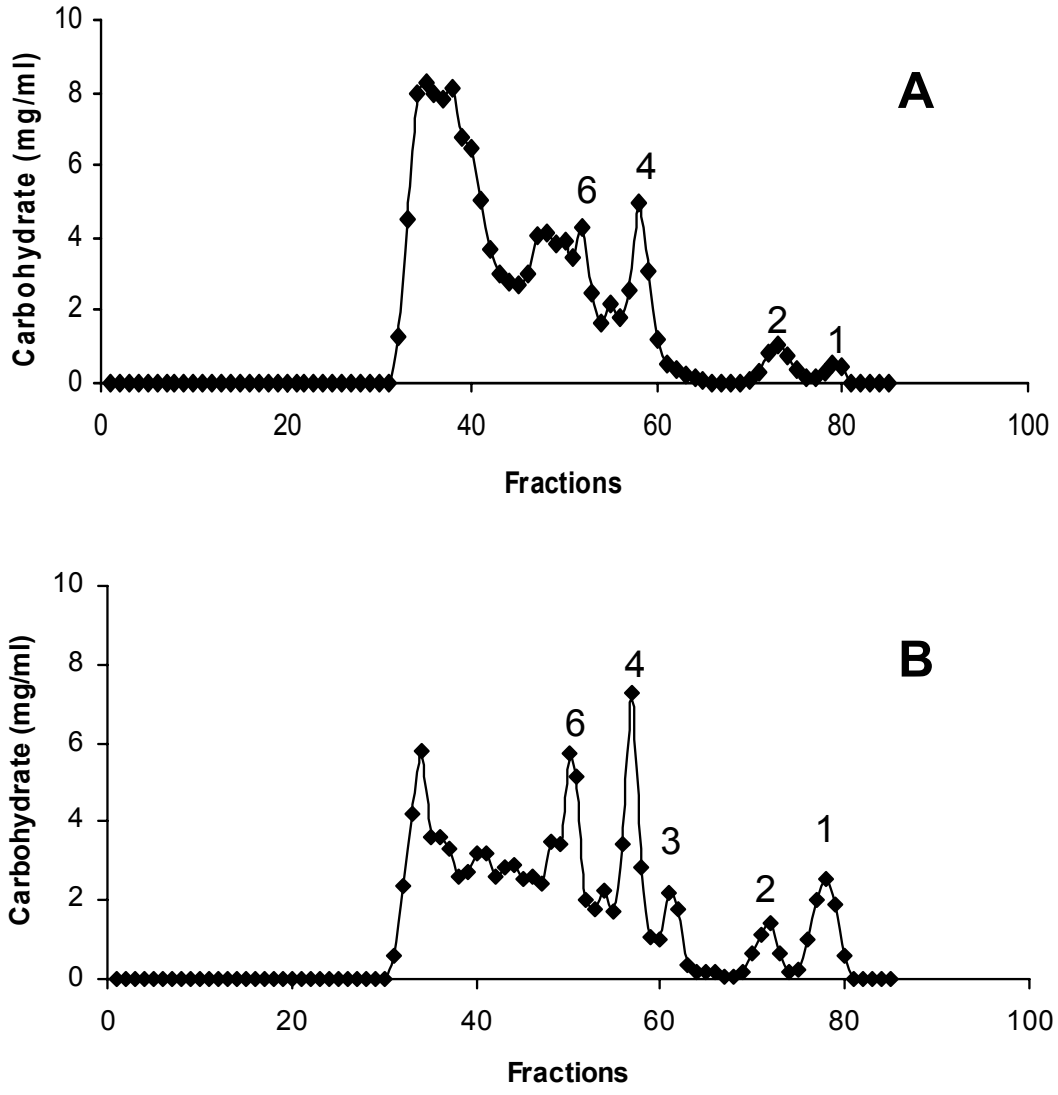

Figure 3: Bio-Gel P2 gel permeation chromatography of the oligosaccharides produced by the hydrolysis of soluble wheat arabinoxylan by *Trichoderma* xylanase M1 (**A**) and by recombinant AXHd3 from *Bifidobacterium adolescentis* followed by *Trichoderma* xylanase M1 (**B**). *Numbers* represent degree of polymerization.

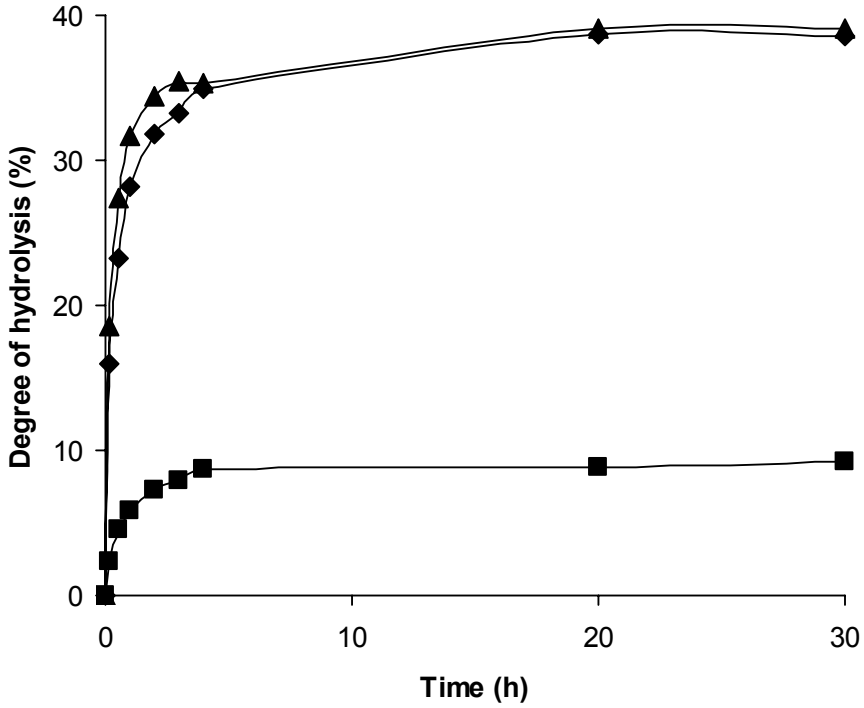

Figure 4: Release of arabinose from wheat arabinoxylan-oligosaccharides by recombinant AXHd3 from *Bifidobacterium adolescentis* (7 units; *squares*), *Aspergillus niger* α-L-arabinofuranosidase (500 units; *diamonds*), or a combination of both enzymes (*triangles*).

DISCUSSION

AXHd3 is a unique enzyme that is able to release only C3 linked arabinose residues from double-substituted xylose residues (Van Laere et al. 1997; 1999) and plays a role in the complete degradation of arabinoxylan and AXOS (Van Laere et al. 1997; 1999). The amino acid sequence revealed that the enzyme belongs to GH family 43, which has an inverting mechanism. This result supports the data obtained by Pitson et al. (1996), who classified AXHd3 as an inverting enzyme on the basis of its lack of glycosyl transfer to methanol.

The highest homology for the deduced amino acid sequence of *axhD3* was found for putative β-xylosidases and β-xylanases. In addition, the highest homology for AXHd3 and the arabinoxylan-degrading enzymes of *B. longum* NCC2705 (Schell et al. 2002) was also found for a putative extracellular exo-xylanase (BL1544; *xynF)*. We propose, that due to this high homology, it

is possibly for these enzymes to be assigned to arabinose releasing-activities instead of β-xylosidase or β-xylanase, moreover because these enzymes are not biochemically characterized yet. Homology for the deduced amino acid sequence of *xylA* was observed with putative xylanases but no homology was found with arabinoxylan-degrading enzymes from *B*. *longum* NCC2705 (Schell et al. 2002). It would be interesting if *xylA* codes for a true xylanase with an endo-activity, as most members of GH-family 8 are glucanases. Intriguingly, the *xylA*-encoded gene product does not contain a signal peptide and it is thus assumed that the enzyme is intracellular. The cloning and biochemical characterization of this enzyme is now in progress.

To date, only a few arabinose-releasing enzymes have been characterized from *Bifidobacterium* spp., despite the presence of 11 genes coding for putative arabinose releasing enzymes in the genome of *B*. *longum* NCC2705 (Schell et al. 2002). The only cloned enzyme is an α-L-arabinofuranosidase from *B*. *longum* B667 (*abfB*), which was active towards polymeric arabinoxylan and released ~25% of the total arabinose residues (Margolles and de Los Reyes-Gavilán 2003). It was concluded that the enzyme exhibited hydrolytic activity against α-1,3 and α-1,5-linked non-reducing terminal L-arabinose residues. However, the activity against α-1,2 linkages could not be excluded. The enzyme was not tested against AXOS, but the activity toward arabinoxylan was rather low. The purified and characterized α-L-arabinopyranosidase and α-L-arabinofuranosidase from *B*. *breve* K110 were not tested for arabinoxylan degrading activity. Arabinogalactan was used as substrate but no activity was observed for either enzyme (Shin et al. 2003b). AXHd3 from *B*. *adolescentis* was active towards arabinoxylan and oligomers derived from it. AXHm23 was only active towards AXOS (Van Laere et al. 1999). The recombinant AXHd3 showed the highest activity towards AXOS.

In general, it was found that AXOS and XOS are better substrates for growth/fermentation than their polymeric precursors or free monosaccharide compounds (Campbell 1997; Crittenden 2002; Jaskari et al. 1998; Okazaki et al. 1990; Van Laere et al. 2000). Fermentation studies of *B*. *longum* co-cultured with *Bacteroides thetaiotaomicron* demonstrated that an arabinogalactan polymer was degraded by *Bact*. *thetaiotaomicron* to oligomers, which subsequently could be utilized by *B*. *longum* (Degnan and Macfarlane 1995). In another study, it was shown that arabinoxylan was degraded by the *Bact*. *fragilis* group and it was postulated that the produced oligosaccharides might provide a carbon source for bifidobacteria (Hopkins et al. 2003). These oligosaccharides might be imported into the cell before hydrolyzing them. In addition, the genome sequence of *B*. *longum* NCC2705 revealed the presence of 8 high-affinity MalEFG-type

oligosaccharide transporters (Schell et al. 2002), more than in any other published prokaryotic genome to date. It is speculated elsewhere that XOS can be transported into certain bacteria, where they are subsequently hydrolyzed and used as a carbon and energy source. For instance, members of the CE family 7 enzymes appear to lack signal peptides; and members of this family have been shown to be acetylated xylo-oligosaccharide esterases, exhibiting no detectable activity on acetylated xylan (Lorenz and Wiegel 1997; Vincent et al. 2003). The degradation of arabinoxylan is therefore assumed to be a co-operative process in the colon. The presence of specific enzymes like AXHd3 shows why *B*. *adolescentis* can utilize AXOS. Also, *B*. *longum* is able to grow on this substrate, an ability presumably enabled by the numerous genes coding for enzymes involved in arabinoxylan degradation (Schell et al. 2002). Interestingly, the absence of arabinoxylan-degrading enzymes in the preliminary genome sequence of *B*. *breve* UCC2003 (D. van Sinderen, personal communication) indicates that AXOS utilization is variable within the genus *Bifidobacterium*.

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Chapter 7

Bifidobacterium carbohydrases

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ABSTRACT

There is an increasing interest to positively influence the human intestinal microbiota through the diet by the use of prebiotics and/or probiotics. It is anticipated that this will balance the microbial composition in the gastrointestinal tract in favor of health promoting genera such as *Bifidobacterium* and *Lactobacillus*. Carbohydrates like dietary fibre and non-digestible oligosaccharides are potential prebiotics. To understand how these bacteria can grow on these carbon sources, knowledge of the carbohydrate-modifying enzymes is needed.

Little is known about the carbohydrate-modifying enzymes of bifidobacteria. Recently, the genome sequence of *Bifidobacterium longum* has been completed and it was observed that more than 8% of the annotated genes were involved in carbohydrate metabolism. Besides, more sequence data of individual carbohydrases from other *Bifidobacterium* spp. became available. Together, these data allow us to pinpoint what kind of poly- and oligosaccharides they can utilize. The carbohydrases of bifidobacteria can also have potential for tailoring more complex or longer carbohydrate structures, at least if they are capable of catalyzing transglycosylation reactions. It is thought that such oligosaccharides can be applied to influence the microbial composition of the more distal parts of the colon. The advantage of using *Bifidobacterium*'s own carbohydrases for this purpose is the increased likelihood that the newly synthesized products can be utilized by this organism. Approaches to use and improve carbohydrate-modifying enzymes in prebiotic design will be discussed.

INTRODUCTION

Bifidobacteria are gram-positive, anaerobic, non-spore-forming, and non-motile bacteria. The rods are often Y- or V-shaped (Scardovi 1986). Tissier (1900) reported in 1900 the isolation of the first bifidobacterium from the intestine of a child, and named it *Bacillus bifidus communis*. The genus *Bifidobacterium* was already recognized by Orla Jensen as a separate taxon in 1924, but it took 50 years before the genus *Bifidobacterium* was for the first time classified in the Bergey's Manual of Determinative Bacteriology (Rogosa 1974). Presently, 34 species (some different biotypes and subspecies) have been described (Chapter 1; Biavati et al. 2000; Hoyles et al. 2002; Masco et al. 2004; Sakata et al. 2002; Simpson et al. 2004; Zhu et al. 2003). The major habitat is considered to be the intestine of man and animals (Biavati et al. 2000) and twelve species have been associated with humans as host. Bifidobacteria rapidly colonize the digestive tract of newly born infants and become the major bacteria in the colon. The number of these known *Bifidobacterium* spp. gradually decreases with age and also the composition of the *Bifidobacterium* spp. is about to change in time (Hopkins et al. 2002; Mitsuoka 1990). Bifidobacteria represents 1-3% of the total fecal bacterial community in adults (Franks et al. 1998; Langendijk et al. 1995).

Definitions of probiotic, prebiotic, and dietary fibre. Carbohydrates play an important role in the gastrointestinal tract (GIT) of humans. Besides their direct physiological effect they also affect the gut ecosystem, which significantly contribute to the well-being of humans (Falk et al. 1998). Bifidobacteria are one of the major groups in the GIT and it is claimed that they have several health-promoting effects (Gibson 1998; Gibson and Roberfroid 1995; Gibson et al. 2000) like preventing diarrhea (Saavedra et al. 1994), lowering cholesterol levels (Beena and Prasad 1997), immunostimulation (Roller et al. 2004), anticarcinogenicity (Burns and Rowland 2000; Reddy 1998; Wollowski et al. 2001), improved mineral absorption (Scholz-Aherns et al. 2002), and production of vitamins (Deguchi et al. 1985). In order to increase the amount of bifidobacteria in the GIT the concepts of probiotics and prebiotics have been developed. To positively influence the microbiota in the GIT probiotics and/or prebiotics can be applied in the diet as a functional food. The definition of a probiotic is 'a preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the micro flora (by implantation or colonization) in a compartment of the host, and by that, exert beneficial health effects in this host' (Schrezenmeir en De Vrese 2001). Bacteria used as probiotic are mainly from the genera *Bifidobacterium* or *Lactobacillus* (O'Sullivan 2001; Tomasik and Tomasik 2003). A prebiotic can be defined as 'a non-

digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that have the potential to improve host health' (Gibson and Roberfroid 1995). The combination of probiotics and prebiotics is termed synbiotic. The definition is 'a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract' (Gibson and Roberfroid 1995). This review will focus on prebiotics and *Bifidobacterium* carbohydrases. For probiotics the reader is referred to other reviews (Kaur et al. 2002; Klaenhammer and Kullen 1999; Schrezenmeir en De Vrese 2001; Stanton et al. 2001); for a critical overview of the health benefits of pre- and probiotics we refer to De Roos and Katan (2000) and Teitelbaum and Walker (2002).

The intake of dietary fibre gives beneficial health effects to humans (Slavin 1999). Trowell et al. (1976) proposed the generally accepted definition of dietary fibre as consisting of the remnants of edible plant cell wall polysaccharides, lignin, and associated substances resistant to (hydrolysis) digestion by the alimentary enzymes of humans. However, a great deal of debate has existed over this definition the last decades. A new definition/description was launched by the American Association of Cereal Chemists (Prosky 2001). Their criteria for dietary fibre were (i) it should be edible parts of plants or analogous carbohydrates, (ii) resistant to digestion and absorption in the small intestine, (iii) complete or partial fermented in the large intestine, (iv) include polysaccharides, oligosaccharides, lignin, and associated plant substances, and (v) promote beneficial physiological effects. Two types of dietary fibre are distinguished; one precipitating in 80% (v/v) ethanol (insoluble dietary fibre), and the other remains in solution in 80% (v/v) ethanol (soluble dietary fibre; Meyer and Tungland 2001). Non-digestible oligosaccharides (NDOs), a functional food ingredient belongs to the soluble dietary fibre. Glycosidases play an important role in the breakdown of NDOs to fermentable sugars. In addition, some glycosidases can also be very useful to synthesize NDOs by their transglycosylase activity in vitro (Voragen 1998).

CLASSIFICATION OF CARBOHYDRASES

Retaining and inverting glycosidases. Enzymatic hydrolysis of glycosidic bonds in oligo- and polysaccharides is carried out with one of two stereo chemical outcomes: net retention or net inversion of the anomeric configuration. Glycosidases are classified as either retaining or inverting

as first proposed by Koshland (1953). Retaining glycosyl hydrolases have a double displacement (S_N1) mechanism (Sinnot 1990) involving a glycosyl-enzyme intermediate. The retaining enzyme has two carboxylic acids in the catalytic center. One carboxylic acid acts first as an acid catalyst and protonates the glycosidic oxygen, while the other carboxylic acid acts as nucleophile and assists departure of the leaving group (Figure 1A). This is the glycosylation step and a glycosyl-enzyme intermediate is formed. Subsequently, the first carboxylic acid will behave as a base catalyst and activate the incoming nucleophile (water), resulting in the hydrolysis of the glycosyl-enzyme intermediate (deglycosylation step). The product formed has the same stereochemistry as the substrate. Generally, the distance of the carboxylic acids is approximately 5.5 Å in retaining enzymes (Davies and Henrissat 1995; McCarter and Withers 1994; Withers 2001). Inverting glycosyl hydrolases have a single displacement (S_N2) mechanism (Sinnot 1990) and have different carboxyl acids acting as acid and base (Figure 1B). In this case the protonation of the glycosidic oxygen and departure of the leaving group are accompanied by a concomitant attack of a water molecule, which is activated by the carboxylic base catalyst. The product has the opposite stereochemistry as the substrate (Davies and Henrissat 1995; McCarter and Withers 1994; Withers 2001). The distance of the carboxylic acids is approximately 6.5–9.5 Å in inverting enzymes.

For retaining enzymes the incoming nucleophile can be a sugar molecule instead of water. This can lead to the formation of oligosaccharides with a higher degree of polymerization or containing a new linkage type. Such reactions are called transglycosylation. Inverting enzymes do not have the capability to synthesize oligosaccharides.

Glycoside hydrolase family classification. In 1991 Henrissat introduced a classification of glycosyl hydrolases based on their amino acid sequence similarities. Glycosyl hydrolases with a high degree of sequence homology were assigned to the same glycoside hydrolase family (GH family; Henrissat 1991). This classification has predictive value with respect to (i) the structural features (fold) of the enzymes, (ii) the evolutionary relationship between the enzymes, and (iii) catalytic mechanism (Henrissat 1991; Henrissat and Bairoch 1993). This classification system is complementary to the International Union of Biochemistry enzyme nomenclature (EC numbers), which is based on substrate specificity of the enzyme (IUB 1984). The database is regularly updated (Henrissat and Bairoch 1993; 1996) and available at internet (http://afmb.cnrs-mrs.fr/CAZY/index.html). Currently, more than 88 GH families are known.

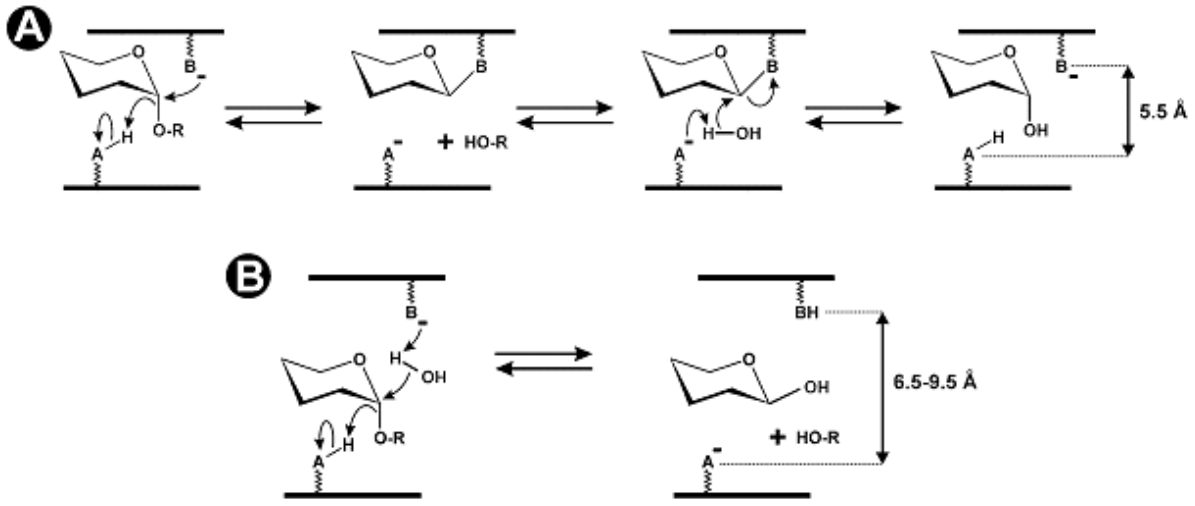

Figure 1: Mechanism of retaining (**A**) and inverting (**B**) glycosyl hydrolases. Details are explained in section Retaining and inverting glycosidases.

Enzymes in one GH family can have different substrate specificity but also a different mode of action (endo or exo). However, it appeared that within a GH family the catalytic mechanism, retaining or inverting, was conserved (Gebler et al. 1992). The catalytic residues are also conserved within a GH family (Henrissat and Bairoch 1996) as well as the protein fold (Davies and Henrissat 1995; Henrissat and Davies 1997). Although only limited information is available about the 3D structures of enzymes from bifidobacteria (Iwata and Ohta 1993; Sprogøe et al. 2004), the 3D structure of more than 51 GH families is known. Some of the GH families are grouped into clans and they have a common ancestry and significant similarities in their tertiary structure. Within a clan the catalytic residues and the catalytic mechanism are conserved (Henrissat and Bairoch 1996). The information in the database can be used to compare the deduced amino acid sequence of genes or for sequenced genomes for searching homologies with similar carbohydrases.

INDUCTION OF ENZYMES BY CARBOHYDRATES

Bifidobacteria play an important role in carbohydrate fermentation in the colon. Oligo- and polysaccharides will be degraded to monosaccharides and these will be converted to intermediates

of the hexose fermentation pathway, also called fructose-6-phosphate shunt or bifid shunt. Subsequently, they will be converted to short chain fatty acids and other organic compounds (De Vries 1969; Scardovi and Trovatelli 1965). In general, gut bacteria degrade carbohydrates to low molecular weight oligosaccharides, which can subsequently be degraded to monosaccharides by the use of a wide range of depolymerizing enzymes. These glycosidases are found extracellularly, associated to the bacterial cell, or intracellularly.

Prebiotics can cause a change in microbial enzyme activity. For example: the activity of β-galactosidase and α-arabinopyranosidase of *B*. *longum* is increased, when growth takes place on larch wood arabinogalactan (Degnan and Macfarlane 1995). In addition, Salyers et al. (1977) and Crociani et al. (1994) found that in *Bifidobacterium* spp. mainly the *B*. *longum* strains were able to grow on arabinogalactan. Growth of *B. adolescentis* on xylose and arabinoxylan-derived oligosaccharides induced the production of two arabinofuranohydrolases (Van Laere et al. 1999b). Also *B*. *longum* produced arabinofuranosidases during growth on arabinoxylan (Crittenden et al. 2002). Sofar, *B*. *longum* and *B*. *adolescentis* seem to be the only *Bifidobacterium* spp. with the ability to grow on arabinoxylan (Crittenden et al. 2002). Preliminary results of the genome sequence of *B*. *breve* UCC2003 revealed that this strain did not contain arabinoxylan-degrading enzymes (Van Sinderen 2004).

Trindade and coworkers (2003) gave another example of an enzyme induced by disaccharides and/or oligosaccharides. They found that sucrase activity of *B*. *lactis* is induced in the presence of sucrose, raffinose, and in small amounts by oligofructose.

The induction of enzymes, which are involved in the degradation of carbohydrates, can be repressed by the presence of glucose (Degnan and Macfarlane 1994; Trindade et al. 2003). This repression of enzyme synthesis is a way of bacteria to control the oligo- and polysaccharide metabolism. When a preferred carbon source is present, there will be no unnecessary production of large amounts of enzyme (Degnan and Macfarlane 1995).

BIFIDOBACTERIUM CARBOHYDRASES

Japanese researchers have performed most of the early work on isolation and characterization of bifidobacteria enzymes (1980–1987; Igaue et al. 1983a; 1983b; Sakai et al. 1986; 1987; Tochikura et al. 1986). An overview of isolated and characterized carbohydrate modifying enzymes is shown

in Table 1. All the enzymes were isolated from *Bifidobacterium* species present in humans except for *B. pseudolongum*.

Initially most of the investigations were directed towards the hydrolytic activity of the enzymes. However, studies with glycosidases showed that some enzymes have transglycosylation activity (for explanation see section Retaining and inverting glycosidases) besides hydrolytic activity. Dumortier et al. (1994) showed that one of the β-D-galactosidases from *B. bifidum* was able to synthesize transgalacto-oligosaccharides (TOS), whereas other purified β-D-galactosidases were not able to produce TOS under the same conditions.

Most of the data about heterologously produced enzymes from *Bifidobacterium* spp. has been published in the last five years; these data are also summarized in Table 1. All enzymes are from bifidobacteria species present in humans, with the exception of those from *B. lactis*, which is commonly found in fermented milk. Most of the carbohydrases are α-galactosidases (GH family 36), β-galactosidases (GH family 2 or 42), and enzymes active against gluco-oligossacharides like α-glucosidases and sucrose phosphorylases (GH family 13).

Since the genome of *B. longum* NCC2705 was published (Schell et al. 2002), more information about carbohydrate modifying enzymes of this organism became available. Five percent of the annotated genes of *B. longum* are carbohydrate modifying enzymes (like glycosyl hydrolases and glycosyl esterases) and carbohydrate binding molecules. For example *L. lactis* contains 2.5% of annotated carbohydrate modifying enzymes, *Clostridium perfringens* 3.1%, *E. coli* K12 1.4%, *Bacillus subtilis* 1.7%, and *Mycobacterium tuberculosis* 1.1% (Henrissat and Couthino 2002). Only *Bacteroides thetaiotaomicron* contains also a high number of annotated carbohydrate modifying enzymes (Xu et al. 2003). In Table 2 an overview is given of annotated carbohydrate modifying enzymes, with a predictive function, of *B. longum* NCC2705. For each enzyme the presence of transmembrane domains (TmD) and/or a signal peptide (SignP) has been indicated, which was based on sequence analysis using SignalP (Nielsen et al. 1997) (http://www.cbs.dtu.dk/services/SignalP-2.0/), PSORT (Nakai and Kanehisa 1991) (http://psort.nibb.ac.jp/), and SOSUI (Hirokawa et al. 1998) (http://sosui.proteome.bio.tuat.ac.jp/cgi-bin/sosui.cgi?/sosuisignal/sosuisignal_submit.html).

TABLE 1: Glycosyl hydrolases from bifidobacteria

Species/strain	Enzyme	Mol. mass (kDa) SDS/native	pH_{opt}	T_{opt} (^{o}C)	pI	Accesion Number	Gene	GH Family	References
B. adolescentis									
	D-xylo-isomerase	53/168	7	60	4.3				Kawai et al. 1994
DSM 20083	AXHd3	–/100	6	30	-				Van Laere et al. 1997; 1999b
		~60/–	6.0	-	-	AF233379	*axhD3*	43	Van den Broek et al. 2005b, chapter 6
	AXHm23	–/160	6	37	-				Van Laere et al. 1999b
	α-glucosidase A	~71/68	6.6	37	-	AF358444	*aglA*	13	Van den Broek et al. 2003, chapter 3
	α-glucosidase B	73/149	6.8	47	-	AF411186	*aglB*	13	Van den Broek et al. 2003, chapter 3
	α-galactosidase	83/330	6	45	-				Leder et al. 1999
		79/344	5.5	55	-				Van Laere et al. 1999a
		83/332	6.5	45	-	AF124596	*aga*	36	Van den Broek et al. 1999, chapter 2
	β-galactosidase	89/350	6	35	-				Van Laere et al. 2000a
		81/235	6.0	50	-	AY359872	*bgal II*	42	Hinz et al. 2004
	sucrose phosphorylase	58/129	6.0-6.5	48	-	AF543301	*sucP*	13	Van den Broek et al. 2004, chapter 4
G1	β-fructofuranosidase	74/75	6.1	-	4.5				Muramatsu et al. 1993; 1994

E194a	α-glucosidase I	97/490	6.0	50	-				Igaue et al. 1983a; 1983b
	α-glucosidase IIa and b	60/120	6.0	50	-				Igaue et al. 1983a; 1983b
Int-57	α-amylase	66/–	5.5	50	5.2				Lee et al. 1997
	β-galactosidase	-	-	-	-	AF213175	*gal*		–
	β-glucosidase	-	-	-	-				Choi et al. 1996
B. animalis* subsp. *lactis									
	sucrose phosphorylase	-	-	-	-	AF441242	*scrP*	13	Trindade et al. 2003
DSM 10140	β-fructofuranosidase	60/60	6.5	40	-	AJ437479	*bfrA*	32	Ehrmann et al. 2003
		60/–	-	-	-	AY509036		32	Janer et al. 2004
B. bifidum									
A3	β-galactosidase	-	7.0	50	-				Iwasaki et al. 1971
DSM 20082	β-galactosidase	163-190/362	6.5	37-39	5.25				Dumortier et al. 1994
	β-1,3-galactosyl-*N*-acetylhexosamine phosphorylase	–/140	6-6.5	40-45	-				Derensy-Dron et al. 1997
DSM 20125	β-galactosidase	–/620	-	-	-	AJ272131	*BIF1*		Møller et al. 2001
		130/236	-	-	-	AJ224434	*BIF2*	2	Møller et al. 2001
		180-360/182	-	-	-	AJ224435	*BIF3*	2	Jørgensen et al. 2003
						AX319625			Møller et al. 2001
JCM1254	1,2-α-L-fucosidase	-	-	-	-	AY303700	*afcA*	95	Katayama et al. 2004

B. breve									
203	α-galactosidase	39/330	5.5	-	3.7				Sakai et al. 1987
		80/160	5.5-6.5	-	-				Xiao et al. 2000
		-	-	-	-	AF406640	*aga*	36	–
	β-glucosidase I	48/96	6.0	45	-				Sakai et al. 1986
	β-glucosidase II	–/450	5.5	40	-				Sakai et al. 1986
203 clb	β-glucosidase	48/47	5.5	45	4.3				Nunoura et al. 1996b; 1996a
		50/50	5.5	45	4.3	D88311		1	Nunoura et al. 1997; 1996a
K-110	α-L-arabinofuranosidase	60/60	4.5	45	-				Shin et al. 2003b
	α-L-arabinopyranosidase	77/310	5.5-6.0	40	-				Shin et al. 2003b
	β-D-xylosidase	49/49	5.0	37	-				Shin et al. 2003a
YIT4010	β-galactosidase	-	-	-	-	E05040			Iono et al. 1993
B. longum* subsp. *infantis									
ATCC15697	β-fructofuranosidase	68/72	6.0	37	4.3				Warchol et al. 2002
	α-galactosidase	-	6.0	40	-				Roy et al. 1992
	β-galactosidase	-	7.0	40	-				Roy et al. 1992
DSM 20088	β-galactosidase	77/140	-	-	-	AJ224436	*INF1*	42	Møller et al. 2001
HL96	β-galactosidase	-	-	-	-	AF192265	*βgalI*	2	Hung et al. 1998; 2001
HL96	β-galactosidase	-	-	-	-	AF192266	*βgalIII*	42	Hung et al. 1998; 2001
JCM 7007	β-fructofuranosidase	75/232	6.0-6.2	55	-				Imamura et al. 1994

B. longum* subsp. *longum									
401	β-galactosidase	–/330	6.0	40	-				Tochikura et al. 1986
	lactase	–/700	6.5	45-50	-				Tochikura et al. 1986
B667	α-L-arabinofuranosidase	-	-	-	-	AY259087	*abfB*	51	Margolles and De Los Reyes-Gavilán 2003
CRL 849	α-galactosidase	-	5.8	40-45	-				Garro et al. 1994
MB219	β-galactosidase	-	-	-	-	AJ242596	*lacZ*	2	Rossi et al. 2000
SJ32	sucrose phosphorylase	56/–	-	-	-	AY236071	*scrP*	13	Kim et al. 2003a
VMKB44	α-galactosidase	-	-	-	-	AF160969	*aglL*	36	–
B. pseudolongum									
NCFB 2244	α-glucosidase	126/126	-	-	4.2				Degnan and Macfarlane 1994

TABLE 2: Overview of some sugar modifying enzymes from *Bifidobacterium longum* NCC2705

Sugar modifying enzyme	BL-number	Glycoside Hydrolyse Family	R/I [a]	TmD and Position TmD (aa)	SignP and Position SignP (aa)
Endogalactanase	257	53	R	Yes, 871-892	Yes, 1-30
β-Fructo-furanosidase	105	32	R	No	No
Endo-α-L-arabinosidase	182	43	I	No	No
Endo-α-L-arabinosidase	183	43	I	No	Yes, 1-31
α-Arabino-furanosidase	181	51	R	No	No
α-L-Arabino-sidase	1611	51	R	No	No
α-L-Arabino-sidase	544	51	R	No	No
α-L-Arabino-furanosidase	1166	51	R	No	No
Exo-α-L-arabinosidase	187	43	I	No	No
α-Galactosidase	1518	36	R	No	Yes/ No [b], 1-37
α-Galactosidase	177	27	R	No	No
β-Galactosidase	259	42	R	No	No
β-Galactosidase	978	2	R	No	No
β-Galactosidase	1168	42	R	No	No
Exoxylanase	1544	43	I	Yes, 935-956	Yes, 1-30
β-Xylanase	1543	43	I	Yes, 815-836	Yes, 1-43
Xylosidase	523	31	R	No	No
β-Glucosidase	1757	2	R	No	No
β-Exoglucanase	1761	3	R	No	No
α-1,4-Glucosidase	529	13	R	No	No
Oligo-1,6-glucosidase	1526	13	R	No	No
Sucrose phosphorylase	536	13	R	No	No
α-Mannosidase	1327	38	R	No	No
α-Mannosidase	1328	38	R	No	No
α-Mannosidase	1329	38	R	No	No

[a] Catalytic mechanism: R is retaining, I is inverting

[b] With SOSUI a signal peptide was found from amino acid 1 to 37. With SignalP and PSORT no signal peptide was found.

Remarkably, only a few enzymes contained a signal peptide, and it is therefore suggested that most carbohydrases are intracellularly located. Only four enzymes were annotated to have an "endo" working mechanism. This shows that *B*. *longum* has probably a preference for oligosaccharides, which are transported into the cell for further utilization. This is in agreement with the presence of a high number of MalEFG-type oligosaccharide transporters identified in the genome of *B*. *longum* (Schell et al. 2002) Also preliminary results of *B*. *breve* UCC2003 revealed a variety of oligosaccharide transporters (Van Sinderen 2004). This indicates that bifidobacteria are very well adapted for the utilization of especially oligosaccharides in the colon

POSSIBLE STRATEGIES OF BIFIDOBACTERIA FOR CARBOHYDRATE UPTAKE

Bacteria can employ different strategies for utilizing carbohydrates as a carbon source. These strategies have in common that they comprise a battery of intra- and extracellular enzymes with activity towards oligo- and polysaccharides, linked to an extensive set of sugar transporters nested in membranes. The genome of *B*. *longum* contains for example eight high-affinity MalEFG-type oligosaccharide transporters and one PTS-type sugar transporter (Schell et al. 2002). However, detailed information regarding the specificity of sugar transporter is mostly lacking. Examples of carbohydrate import by *Bifidobacterium* described in some detail include that of glucose and arabinose in *B*. *breve* (Degnan and Macfarlane 1993), lactose, glucose, and galactose in *B*. *bifidum* (Krzewinski et al; 1996; 1997), lactose and glucose in *B*. *longum* (Kim et al. 2003b), and of galacto-oligosaccharides (Gopal et al. 2001) and sucrose in *B*. *lactis* (Trindade et al. 2003). It is also hypothesized that some *Bifidobacterium* species are able to import xylo-oligosaccharides (Crittenden et al. 2002).

Below, we will discuss the mechanisms of starch utilization of *Bact*. *thetaiotaomicron* (Figure 2). We hypothesize that *B*. *longum* may use a strategy for (arabino)xylan (a dietary fibre) utilization, which is reminiscent of that of *Bact*. *thetaiotaomicron*.

Bact. *thetaiotaomicron* makes use of a kind of docking station to capture starch molecules and bind these to the bacterial cell surface without loosing them to nearby competitors (Hooper et al. 2002). The docking station is a complex of proteins, which is present in the outer membrane of the bacterium. Proximal to this complex can be α-amylase, which is anchored to the cell surface by

a TmD. The enzyme degrades the starch molecule to linear maltodextrins, sufficiently small to pass through a porin which gives access to the periplasmic space. Subsequently, the oligosaccharides are further degraded and internalized by an unknown mechanism.

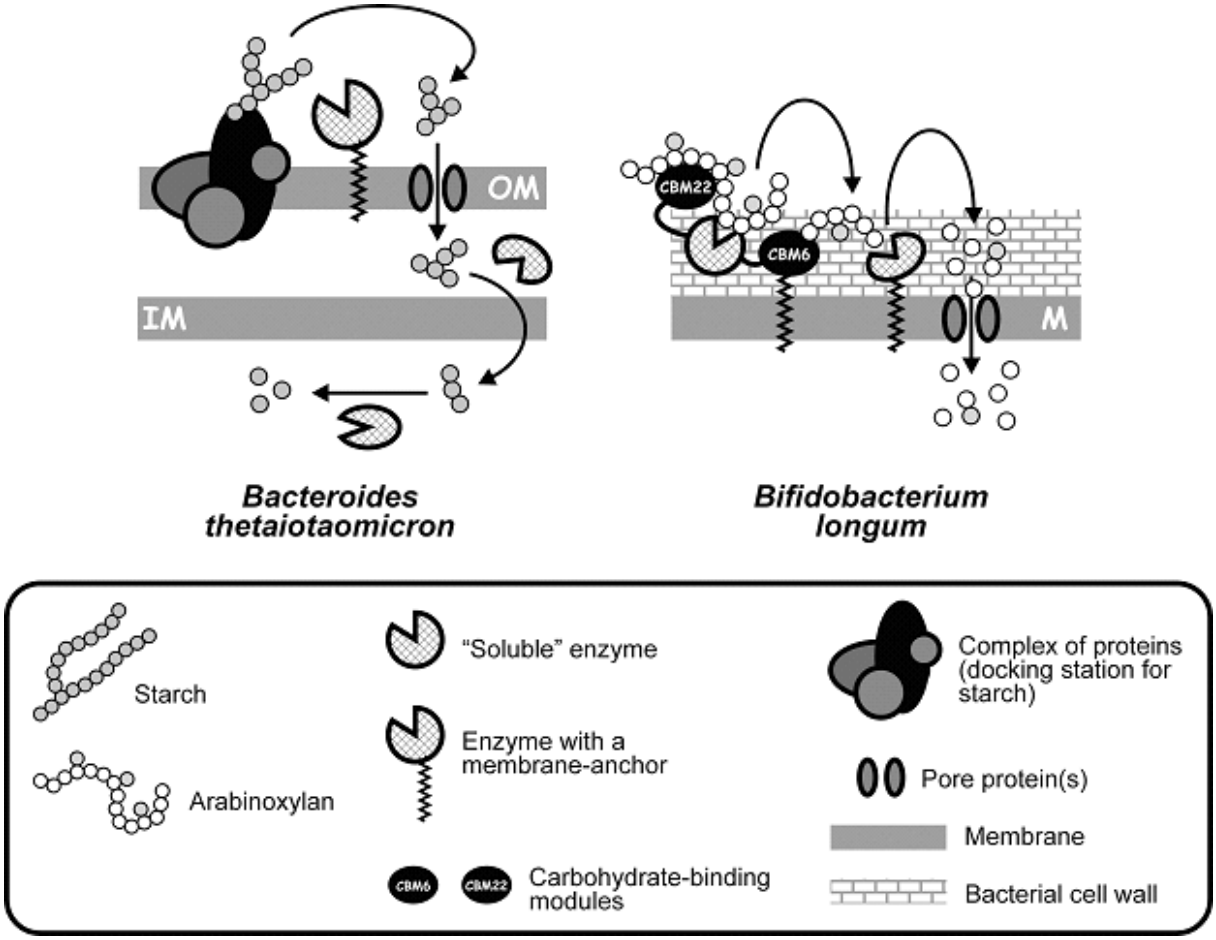

Figure 2: Schematic illustration of different (putative) strategies adopted by various microbes to secure carbohydrate nutrients for their own benefit. IM = inner membrane; OM = outer membrane; M = membrane.

The genome sequence of *B. longum* indicates that this organism is equipped with a number of modular glycanases. A few examples of such enzymes will be discussed, together with their putative role in (prebiotic) carbohydrate utilization. The most striking multi-domain glycanase

seems to be the putative endo-xylanase (BL1543) SignP–CBM22–GH43–CBM6–TmD (CBM=carbohydrate-binding module; Figure 2). The SignP indicates that this enzyme is secreted, whereas the TmD indicates that the enzyme is anchored in the cell membrane. The catalytic domain belongs to GH family 43, and shows homology to other endoxylanases. The exact substrate specificity of the GH43 domain is unknown, i.e. it is not known whether the enzyme is hindered by the presence of arabinosyl substituents as in arabinoxylans. For this reason it is not possible to state with certainty that arabinoxylan can be utilized by *B. longum*. Carbohydrate-binding modules, CBM6 and CBM22, flank the GH43 domain. Other CBMs belonging to these protein families have been shown to interact with xylan. Therefore, we postulate that the two CBMs may function as a kind of docking station, which is an intrinsic part of the endoxylanase, contrary to the machinery of *Bact. thetaiotaomicron* in which the docking station and the hydrolase are separate proteins. Interestingly, *B. longum* also seems to contain an extracellular exo-xylanase (BL1544), which is also equipped with a TmD. It is possible that this enzyme degrades the products of the endo-xylanase further to monosaccharides, which are subsequently transported over the bacterial membrane by a transporter protein that remains to be identified. However, it is also possible that the exo-xylanase serves another purpose, because Crittenden and coworkers (2002) observed that *B. longum* strains grew well on xylo-oligosaccharides (dp 2-5) and much less on xylose. The presence of the bacterial cell wall may prohibit diffusion of these nutrients away from the transporter. The fate of arabinosylated xylo-oligosaccharides, which are also expected to be formed by the endo-xylanase, is unknown. They may be lost as a nutritional source; alternatively, they may be internalized by di- or trisaccharide transporters, after which they are further degraded by arabinofuranosidases inside the cell. The genome sequence of *B. longum* has not revealed extracellular arabinofuranosidases to degrade arabinoxylan-oligosaccharides further (Table 2).

Besides, a putative system for (arabino)xylan utilization, it is likely that *B. longum* can also thrive on (arabino)galactan. From fermentation studies it is known that *B. longum* can utilize arabinogalactan (Crociani et al. 1994; Degnan and Macfarlane 1995; Salyers et al. 1977). The genome sequence reveals the presence of a membrane-bound, extracellular endogalactanase. Typically, *B. longum* does not appear to have an extracellular β-galactosidase. This may indicate that this microorganism has a kind of mechanism to internalize galacto-oligosaccharides, which has also been suggested for *B. lactis* based on growth studies with these oligosaccharides (Gopal et al. 2001). It is also unclear whether galacto-oligosaccharides with arabinosyl side chains (formed upon arabinogalactan degradation) can be taken up by *B. longum*. Table 2 summarizes a number of enzymes, which may have a role in removing arabinosyl substituents. The enzyme annotated as an

endo-α-L-arabin*osidase* (BL183), which is equipped with a signal peptide sequence, may be involved in this process, because the genome sequence has not revealed any other extracellular arabinofuranosidases or endo-arabinanases. The exact mechanism of action remains to be established, since the prefix "*endo*" suggests that the enzyme acts randomly on arabinans, whereas the ending "*osidase*" suggests an exo mode of action. The fact that the enzyme is classified in GH family 43 is not conclusive on its mode of action, because both endo- and exo-enzymes have been assigned to this GH family.

Import of larger galacto-oligosaccharides is not necessarily a common feature of bifidobacteria. For instance, Møller et al. (2001) described a β-galactosidase (BIF3) from *B. bifidum* having a SignP, which suggests that this protein is secreted by the bacterium. Besides, the enzyme contained a C-terminal CBM with high homology to cell surface-attached galacto-binding domains, sometimes referred to as lectins. We speculate that this enzyme will be attached to carbohydrates of the *Bifidobacterium* cell wall through the galactose-recognizing CBM, because galactose is an important constituent glycosyl residue in the backbone of *Bifidobacterium* cell wall polysaccharides, and the predominant residue in their (single unit) side chains (Nagaoka et al. 1995; 1996). If this β-galactosidase is located extracellularly and attached to the cell wall, oligosaccharides will be degraded by the β-galactosidase to monomers. In that case no special oligosaccharides-transporter will be needed, but galactose may be internalized through the more common hexose transporters.

BIFIDOBACTERIUM CARBOHYDRASES FOR OLIGO-SACCHARIDE UTILIZATION AND PRODUCTION

A whole range of carbohydrates has been tested for use as prebiotic like fructo-oligosaccharides, β-galacto-oligosaccharides, α-galacto-oligosaccharides, α-gluco-oligosaccharides, and lactulose. The prebiotic 'effect' was studied in most cases by (i) fermentation of the carbohydrates by bifidobacteria (Crociani et al. 1994; Salyers et al. 1977) and/or (ii) the enumeration of bifidobacteria was investigated in human and animal studies after intake of the carbohydrates (Gibson et al. 2000). In this review, we want to pay attention to another way of examining the prebiotic effect of carbohydrates by showing the presence and activity of carbohydrate modifying enzymes in bifidobacteria. Here we give some examples, which are based on the presence of carbohydrases.

Fructo-oligosaccharides. The application of fructo-oligosaccharides (FOS) as prebiotic ingredient in dairy products and other foods such as bread, fruit preparations, and meat products is increasing (Francks 2000). FOS is the best studied prebiotic to date. It is present in nature in various plant sources (i.e. onion and chicory) or can be prepared from sucrose through the transfructosylation action of enzymes, namely β-fructofuranosidase and β-D-fructosyltransferase. FOS have the general structures (β-D-Fru*f*-(2→1))$_n$-β-D-Fru*f*-(2↔1)-α-D-Glc*p* and (β-D-Fru*f*-(2→1))$_n$-D-Fru*f*. Inulin is the polymeric form of the former. Bifidobacteria are known to ferment FOS rapidly (Durieux et al. 2001), making use of the enzyme β-fructofuranosidase. In a FOS-mixture the shorter oligosaccharides are first utilized by bifidobacteria (Janer et al. 2004; Perrin et al. 2001). Well-described *Bifidobacterium* β-fructofuranosidases are those from *B*. *adolescentis* (Muramatsu et al. 1992; 1993; 1994), *B. infantis* (Warchol et al. 2002), and *B*. *lactis* (Ehrmann et al. 2003; Janer et al. 2004). Muramatsu et al. (1994) differentiate β-fructofuranosidases into those active towards sucrose, inulin, and FOS. Their β-fructofuranosidase from *B*. *adolescentis* G1 was purified and appeared rather specific for fructo-oligosaccharides. Although hydrolysis is 63-times more effective than transglycosylation, some transglycosylation products with β-D-Fru*f*-(2→1)-β-D-Fru*f*-(2↔1)-α-D-Glc*p* (GF_2) as substrate were formed. Products obtained were GF_3, neokestose (β-D-Fru*f*-(2↔6)-α-D-Glc*p*-(1↔2)-D-Fru*f*), and β-D-Fru*f*-(2→1)-β-D-Fru*f*-(2↔6)-α-D-Glc*p*-(1↔2)-D-Fru*f*.

A β-fructofuranosidase with almost the same physico-chemical properties was isolated from *B*. *infantis* ATCC15697 (Warchol et al. 2002), and the enzyme showed also preference for the hydrolysis of oligosaccharides (over that of sucrose and inulin). This β-fructofuranosidase was inactive towards raffinose, which shows that it is different from a heterologously expressed β-fructofuranosidase from *B*. *lactis* DSM10140 that was able to release fructose at a low rate from this substrate (Ehrmann 2003). No secretion or membrane-anchoring sequences were identified suggesting that the protein is located intracellularly. The highest activity was found with sucrose, while fructo-oligosaccharides were hydrolyzed at a slower rate. However, recently another group cloned the same gene and they reported highest activity toward FOS instead of sucrose (Janer et al. 2004). Bifidobacteria can also degrade levan oligosaccharides (β-(2→6)-linked fructose oligosaccharides; Marx et al. 2000).

β-Galacto-oligosaccharides. β-Galactosidases are essential enzymes for bifidobacteria to be able to grow on milk or milk-based substrates such as lactose and lactose-derived transgalacto-oligosaccharides (TOS). A diversity of *Bifidobacterium* strains has been studied with respect to their β-galactosidase activity, in more or less detail. These strains include *B*. *longum* (Tochikura et

al. 1986), *B. infantis* (Hung and Lee 1998; 2002; Hung et al. 2001; Møller et al. 2001; Roy et al. 1992), *B. bifidum* (Dumortier et al. 1994; Garman et al. 1996; Iwasaki et al. 1971; Jørgensen et al. 2001; Møller et al. 2001), *B. adolescentis* (Van Laere et al. 2000a), and *B. lactis* (Gopal et al. 2001). Initially these studies on β-galactosidases focused on the hydrolytic degradation of lactose but gradually more and more attention was paid to their transferase activity towards lactose, for the synthesis of TOS. In this respect also *B. angulatum* and *B. pseudolongum* can be mentioned as sources for β-galactosyl-transferring β-galactosidases (Rabiu et al. 2001). Obviously, the strains with the transglycosylating β-galactosidases also showed the ability to hydrolyze the galacto-oligosaccharides again.

The genomic sequence of *B. longum* NCC2705 revealed the existence of multiple forms of β-galactosidases in this species (Schell et al. 2002); these enzymes belong to GH family 2 or 42 (Table 1). In a crude extract of *B. infantis* ATTC 27920 and HL96 three different β-galactosidase bands were observed upon native electrophoresis (Hung and Lee 1998; Roy et al. 1992). Two β-galactosidase genes from *B. infantis* HL96 (β-GalI and β-GalIII; Hung and Lee 1998; 2002; Hung et al. 2001) and one from *B. infantis* DSM20088 (INF1; Møller et al. 2001) have been cloned. With respect to the amino acid sequence β-GalIII shows 74% identity with INF1. There is a striking difference in their transglycosylation activity. β-GalI is six times more effective in transferring galactosyl groups to lactose than β-GalIII, resulting in the product β-Gal-(1→3)-β-Gal (1→4)-Glc (3'-galactosyl lactose).

Several investigators describe β-galactosidases from *B. bifidum* displaying transferase activity (Dumortier et al. 1990; 1994; Garman et al. 1996; Jørgensen et al. 2001; Møller et al. 2001). Dumortier et al. (1994) purified a transgalactosidase (TG-ase) and from a diversity of monomeric sugars only glucose and xylose could act as acceptors for TG-ase, using lactose as donor. Galactose was only an acceptor when present in a glycosidic linkage, i.e. α-methyl–D-galactose and lactose. Three other genes from *B. bifidum* DSM20215 cloned and expressed in *E. coli* resulted in β-galactosidases named BIF1, BIF2, and BIF3. Only BIF3 was likely to be extracellular, since it contained a signal peptide. Besides hydrolysis, all showed transferase activity with lactose as substrate. The efficiency of transferase activity of BIF3 could be increased tremendously by truncation of this β-galactosidase at the C-terminal end by 580 amino acids (Jørgensen et al. 2001). This C-terminal end contains a galactose binding domain. In *Bact. thetaiotaomicron* also a β-galactosidase was found, which contained a similar galactose binding domain (Xu et al. 2003). Although with many β-galactosidases transfer reactions are only observed with a high lactose

concentration, the truncated form of BIF3 resulted in 90% transfer and only 10% hydrolysis over a wide range of lactose concentrations (10-40% lactose). The molecular mechanism behind this increase in transferring power of the enzyme is yet unknown. Jørgensen et al. (2001) hypothesized that the truncated β-galactosidase may have a more open structure which facilitates transglycosylation. To compare the β-galactosidases genes a dendrogram was constructed (Figure 3). The β-galactosidase genes from the *B*. *longum* genome, annotated by Schell et al. (2002), were included in this dendrogram as well. Two groups of β-galactosidases were found, namely the β-galactosidases from GH family 2 and the β-galactosidases from GH family 42. The enzymes from GH family 2 have different properties than the one from GH family 42. The members of GH family 2 have a higher lactase activity and higher transferase activity than the β-galactosidases from GH family 42 (Hung et al. 2001; Møller et al. 2001). If only the catalytic domain is used to construct the phylogenetic tree, BIF3 was clustered in GH family 2; otherwise it was not clusterd in these two GH families. It can be expected that this enzyme will have a different behavior than the other GH family 2 members.

In *B*. *adolescentis* DSM20083 the presence of at least two β–galactosidases was demonstrated by native gel electrophoresis (β-Gal I and β-Gal II), using 4-methylumbelliferyl-β-galactoside as substrate (Van Laere et al. 2000a). β-Gal I was a typical lactose hydrolyzing enzyme, while β-Gal II appeared unable to do so. Growth of *B*. *adolescentis* on TOS appeared to be a 'two-phase' process. In the first phase lactose was utilized (by β-Gal I) until the cell density reached a plateau level, followed by a second phase in which larger oligosaccharides were fermented and the formation of β-gal II was observed. Experiments with a heterologously produced β-Gal II showed that this enzyme belongs to GH family 42 and showed preference for β-(1-4)-galactosides, such as in arabinogalactan-oligosaccharides derived from potato galactan instead of lactose (Hinz et al. 2004).

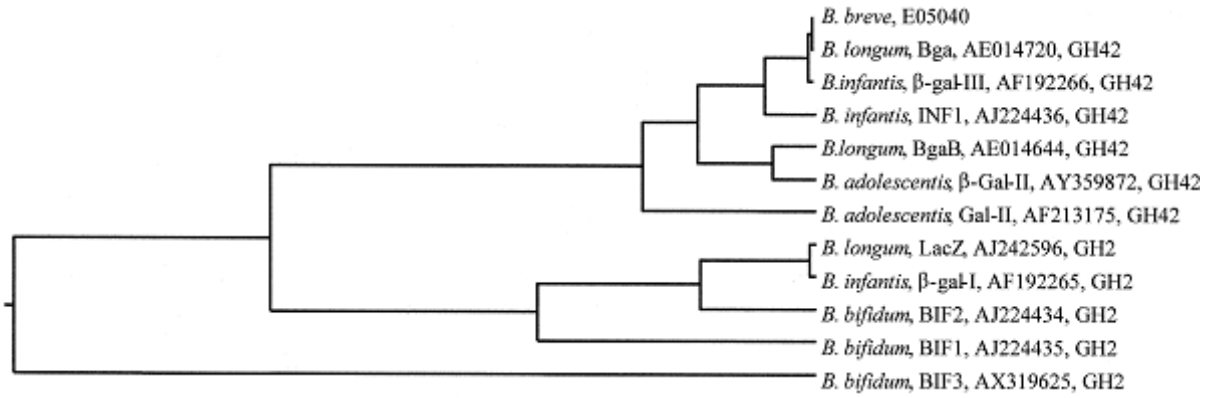

Figure 3: Phylogenetic dendrogram of β-galactosidases from *Bifidobacterium* spp .was constructed using Clustel W. GH 42 or 2 is glycoside hydrolase family 42 or 2, respectively.

The concept of using strain-specific β-galactosidases for the production of oligosaccharides, with a prebiotic function for that specific strain, was nicely demonstrated for a series of *Bifidobacterium* strains (Rabiu et al. 2001). β-galactosidase extracts were used to produce transgalacto-oligosaccharides with different linkage compositions from lactose. As a general rule the specific strain showed the highest growth rate on the oligosaccharide mixture produced by its own β-galactosidase. Only the *B. adolescentis* ANB-7 strain was an exception to this rule. In addition, for the development of a synbiotic, Lamoureux et al. (2002) used mixed cultures of bifidobacteria in the preparation of yoghurts to produce oligosaccharides in these yoghurts.

Arabinan, arabinogalactan, and arabinoxylan. Arabinofuranose-containing oligosaccharides derived from plant cell wall polysaccharides arabinan, arabinogalactan, and arabinoxylan by endo-cleaving enzymes can be fermented by bifidobacteria (Van Laere et al. 2000b). In these oligosaccharides arabinose is mainly present as single unit side chains. The first well-characterized enzymes from *Bifidobacterium* spp. able to degrade arabinoxylan and arabinoxylo-oligosaccharides have been described by Van Laere and coworkers (1997; 1999b). Two different arabinofuranosidases were purified from a cell-extract of *B. adolescentis* DSM20083. Both enzymes were very specific for arabinoxylan (or oligosaccharides thereof, see further). Therefore, these enzymes were named arabinoxylan arabinofuranohydrolases (AXH). A further specificity was observed when preference for the glycosidic linkage type was investigated. In this way an AXHd3 and an AXHm23 could be distinguished. AXHd3 hydrolyzed only C3 linked arabinofuranosyl

residues of double substituted xylopyranosyl residues of arabinoxylan or oligosaccharides thereof. AXHm23 released only arabinosyl units that were linked to the C2 or C3 position of single substituted xylose residues in arabinoxylo-oligosaccharides (Figure 4). Both enzymes were induced specifically by arabinoxylo-oligosaccharides containing doubly substituted xylopyranosyl residues. Besides AXHd3 and AXHm23 also a β-xylosidase active towards linear xylo-oligosaccharides is produced by *B. adolescentis* DSM20083, enabling complete degradation of the branched oligosaccharides to monosaccharides.

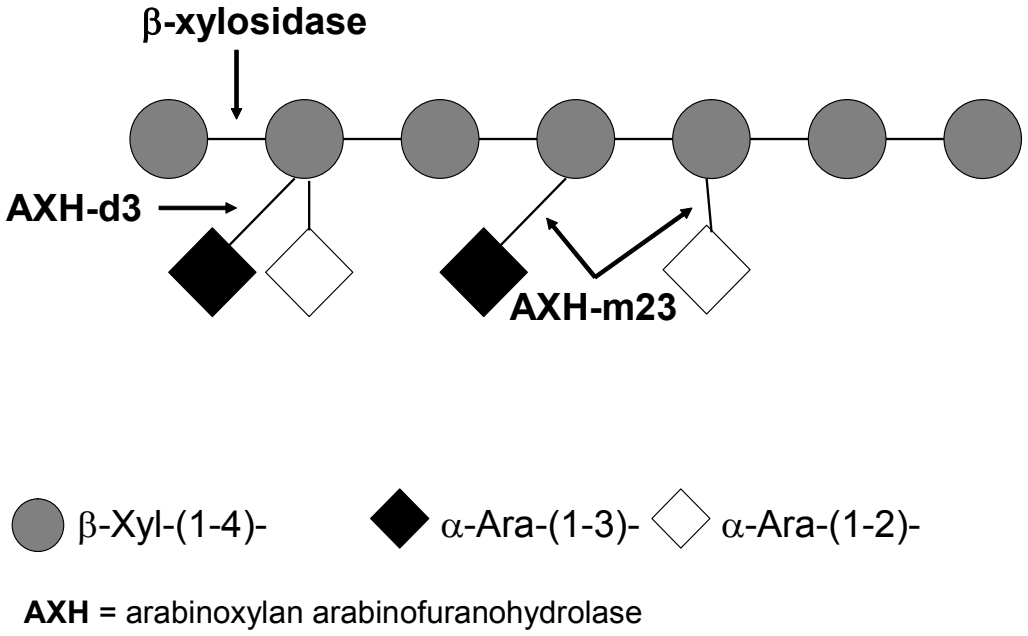

Figure 4: Schematic illustration of the different mode of action of various arabinosyl-removing enzymes on arabinoxylan. Xylosyl residues can carry arabinosyl substituents at the C2 and C3 position.

The relatively large number of genes coding for putative arabinoxylan degrading enzymes in *B. longum* NCC2705 (Table 2; Schell et al. 2002) demonstrates also the importance of these enzymes for survival of this microorganism in its environment. The deduced amino acid sequence of an α-L-arabinofuranosidase gene (*abfB*) from *B. longum* B667 (Margolles and De Los Reyes-Gavilán 2003) showed more than 99% identity to the deduced amino acid sequence of one of the *B. longum* NCC275 arabinosidase genes (NC004307; BL1166). The enzyme showed only exo-activity and broad substrate specificity when compared to AXHd3 and AXHm23 from *B. adolescentis*. Shin et al. (2003b) investigated an α-L-arabinopyranosidase and an α-L-arabinofuranosidase from *B. breve* K110*,* but were not able to detect activity towards arabinogalactan from larch wood. These enzymes were shown to be important for bioconversion of two types of glycosylated ginsenosides named Rb2 and Rc. In a parallel study by the same investigators a β-D-xylosidase was purified from *B. breve* K110 (Shin et al. 2003a). Besides *p*-nitrophenyl-β-D-xylose hydrolyzing activity this enzyme also released xylose from xylan and from the ginsenosides Ra1 and Ra2, which are the β-D-xylosylated forms of Rb2 and Rc, respectively. In contrast, preliminary results of the genome sequence of *B. breve* UCC2003 showed the absence of arabinoxylan degrading enzymes (Van Sinderen 2004). It is concluded that arabinoxylan-oligosaccharides have some selectivity within the genus *Bifidobacterium*, and is supported by in vitro fermentation studies of arabinoxylan by Crittenden and coworkers (2002).

Fermentation studies of bifidobacteria with arabinoxylan and arabinogalactan revealed that the rate of degradation of these polymers is rather low contrary to the oligosaccharides derived thereof. It was assumed that, despite the high number of glycosidases, other microorganisms are needed for the hydrolysis of polymeric arabinoxylan and arabinogalactan into oligosaccharides (Degnan and Macfarlane 1995; Hopkins et al. 2003). Feed-crossing by e.g. *Bact. thetaiotaomicron* could be possible, because most of their glycosyl hydrolases are predicted to be extracellular (Xu et al. 2003). In addition, the *B. longum* genome contained mainly glycosyl hydrolases that did not have a signal peptide for extracellular secretion (Table 2; Schell et al. 2002).

α-Galacto-oligosaccharides and galactomannan. Bifidobacteria are known to grow very well on α-galactosyl oligosaccharides from soymilk such as raffinose and stachyose (Garro et al. 1999; Minami et al. 1983; Yazawa et al. 1978). The α-galactosidase responsible for the degradation of these types of substrates has been studied for *B. breve* (Sakai et al. 1987; Xiao et al. 2000), *B. infantis* (Roy et al. 1992), *B. longum* (Garro et al. 1994), and *B. adolescentis* (Chapter 2; Leder et al. 1999; Van den Broek et al. 1999; Van Laere et al. 1999a). Besides raffinose and stachyose, also

melibiose is easily degraded by these α-galactosidases. Sakai et al. (1987) mentioned the release of galactose from galactomannan.

Relatively much information is available about the α-galactosidase from *B. adolescentis* (Chapter 2; Leder et al. 1999; Van den Broek et al. 1999; Van Laere et al. 1999a). The α-galactosidase was tested on several substrates and was found to be active toward melibiose, α-D-Gal*p*-(1→3)-D-Gal*p*, raffinose, α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc*p*, stachyose, and verbascose. In contrast to the α-galactosidase from *B. breve* (Sakai et al. 1987), this α-galactosidase was unable to act on galactomannan or oligosaccharides thereof. Besides hydrolytic activity, the *B. adolescentis* α-galactosidase showed strong transgalactosylation activity. Starting from melibiose, raffinose, or stachyose elongated oligosaccharides could be formed. The structure of the synthesized products from melibiose were determined and appeared to be the trisaccharide α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-D-Glc*p* and the tetrasaccharide α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-D-Glc*p*. So, it was concluded that selective transgalactosylation at the C6-hydroxyl group took place (Van Laere et al. 1999a). Similar results were found for the heterologously produced enzyme (Chapter 2; Van den Broek et al. 1999). In contrast to the preferred formation of 1→6 linkages during the transfer reaction, Leder et al. (1999) showed that 1→3 linkages are hydrolyzed at a higher rate than 1→6 linkages. Based on the amino acid sequence (Chapter 2; Van den Broek et al. 1999), the α-galactosidase was classified in GH family 36, which is consistent with the observed retention of configuration of the galactose residue during hydrolysis (α→α).

Starch and α-gluco-oligosaccharides. Although bifidobacteria are known to utilize starch (Crociani et al. 1994), there is only one report in the literature that describes the purification of an α-amylase, i.e. from *B. adolescentis* Int-57 (Lee et al. 1997). More research is published about α-glucosidases that are able to hydrolyze oligosaccharides derived from starch and other α-glucosides. From *B. adolescentis* type a, E194a at least two different α-glucosidases were purified (type I and types IIa and IIb) using maltitol and maltose as substrates, but none of them was able to degrade starch (Igaue et al. 1983a; 1983b). Type I was relatively specific for nigerose, kojibiose, and maltitol, while sucrose was not hydrolyzed; on the other hand types IIa and IIb showed broader substrate specificity, including degradation of sucrose. Also Van den Broek and coworkers (2003) demonstrated the existence of at least two different α-glucosidases in *B. adolescentis* DSM20083 (Chapter 3), the cloned genes were named *aglA* and *aglB*, both belonging to GH family 13. The enzymes showed strong activity towards isomaltose (α-D-Gluc*p*-(1→6)-α-D-Gluc*p*) but not towards

starch. With respect to substrate specificity AglA was different from type I, IIa, and IIb α-glucosidase from *B. adolescentis* type a, E194a, while AglB resembles types IIa and IIb. AglB could play a role in the utilization of isomalto-oligosaccharides that can be used as prebiotic, because the enzyme showed high activity toward isomaltotriose (Chapter 3; Van den Broek et al. 2003). Two cell wall associated α-glucosidases were identified and partially purified from *B. pseudolongum*, but none of them was able to hydrolyze starch. Also the genomic sequence of *B. longum* does not indicate the presence of an α-amylase in that strain (Schell et al. 2002) and only one α-glucosidase was reported. In contrast to these findings Wang et al. (1999) showed multiple forms of α-amylase in extracts from *B. bifidum* and *B. pseudolongum*, using SDS-PAGE and reactivation of the denatured enzymes.

In parallel to the other glycosidases described above, also the α-glucosidases from *B adolescentis* DSM20083 showed transferase activity (Chapter 3; Van den Broek et al. 2003). AglA produced oligosaccharides from trehalose and sucrose, while AglB was able to synthesize oligosaccharides from maltose, sucrose, and melizitose. The structures of the newly formed products were not determined, but it can be assumed that they were obtained by α-glucosyl transfer.

Sucrose phosphorylase is another enzyme in *B. adolescentis* that is able to catalyze α-glucosyl transfer (Chapter 4; Van den Broek et al. 2004). This enzyme (SucP) belongs to the same GH-family as the α-glucosidases (GH family 13). SucP can also perform transglucosylation with glucose 1-phosphate as donor and a large number of monomeric sugars (or their alditols) as acceptors. Oligosaccharides could not serve as acceptor substrate. The transglucosylation product with D-arabinose as acceptor was determined to be a novel non-reducing disaccharide with the structure α-Gluc*p*(1↔1)β-Ara*f*. In chapter 5 the crystal structure of SucP was determined (Sprogøe et al. 2004).

Sucrose phosphorylase produces glucose 1-phosphate from sucrose and free phosphate in the cell. This reaction is energetically advantageous, because it bypasses the ATP-requiring step of the hexokinase reaction to phosphorylate glucose in preparation for glycolysis. This way sucrose phosphorylase can play an important role in the fermentation of sucrose obtained after hydrolysis of i.e. raffinose, stachyose, and fructo-oligosaccharides. The sucrose phosphorylase gene was also cloned from *B. longum* (Kim et al. 2003) and *B. lactis* (Trindade et al. 2003) and for the enzyme of *B. lactis* it was shown that the enzyme production was repressed by glucose.

β-Glucosides. Conversion of β-glucosides like cellobiose or plant glycosides (Choi et al. 1996) in the human gut is a well-known process. The β-glucosidases from *B. breve* 203 and *B. breve* clb

(which was derived from strain 203 after acclimation to cellobiose; Nunoura et al. 1996b; 1997b) have been studied extensively (Sakai et al. 1986; 1989). The purified β-glucI from *B*. *breve* clb was highly active towards β1→3 linkages (laminaribiose), β1→2 linkages (sophorose), and β1→4 linkages (cellobiose), but was almost inactive towards β1→6 linkages (gentiobiose). Remarkably, the high activity towards laminaribiose was not observed for β-glucI from the original 203 strain, so some modification of the enzyme must have taken place during acclimation. Using *p*-nitrophenyl-β-D-fucose as donor and glucose as acceptor, four different products were formed with β-glucI from *B*. *breve* clb (Sakai et al. 1989). These were identified as fucosylglucose with the four possible different linkages (β1→2, 3, 4, and 6). These oligosaccharides were very selectively assimilated by bifidobacteria. The heterologously produced β-GlucI (Nunoura et al. 1996a) was used for the production of oligosaccharides by a condensation reaction instead of a transfer reaction. Condensation is the reverse reaction of hydrolysis. Two major oligosaccharides were obtained upon incubation with β-glucI and D-glucose and D-fucose, which were identified as β-D-Fuc*p*-(1→6)-D-Glc*p* and β-D-Glc*p*-(1→6)-D-Glc*p* (gentiobiose; Nunoura et al. 1997a) The dimers were again selectively assimilated by several *Bifidobacterium* strains. The condensation reaction (Suganuma et al. 1978) is probably less energetically favorable than the transglycosylation reaction.

FUTURE DIRECTIONS

Over the years, various approaches have been used to improve the interaction between bifidobacteria and their potential substrates. A striking example of this are the efforts of *Bifidobacterium* strain improvement by chemical mutagenesis, which was aimed at creating probiotic strains with improved lactose utilization (Ibrahim and O'Sullivan 2000). Another approach could be the introduction of foreign DNA into *Bifidobacterium* strains, although this still requires the development of efficient transformation protocols (Van der Werf and Venema 2001). Such approaches may hold potential in the long run, at least when they are accompanied by extensive safety studies and carefully consider consumer acceptance. These two approaches will not be discussed further here, but rather we focus on (i) strategies for efficient prebiotic oligosaccharide production using *Bifidobacterium*'s own carbohydrases, and (ii) exploiting *Bifidobacterium*'s genome sequence for rationalizing prebiotic development.

Strategies for efficient prebiotic oligosaccharide production. It has been suggested above that the carbohydrases of bifidobacteria (or, more in general, beneficial intestinal microbes) can be used for the production of prebiotic oligosaccharides, at least when these enzymes have transglycosylation activity. The rationale behind this is that oligosaccharides can be obtained, that can easily be utilized by bifidobacteria, because all the tools for degradation and uptake are ready to use. The transglycosylation reactions proceed most favorably at high substrate concentration, making it desirable that the substrate is highly water-soluble, or that the reaction can take place at elevated temperatures. Usually, rather cheap oligosaccharides or sugars, such as sucrose and lactose, serve as a starting point for chain elongation. These synthesized oligosaccharides might be incorporated in food products to influence the microbial composition in the more distal parts of the colon (Voragen 1998), because most of the gut disorders are taking place in that region of the colon (Gibson et al. 2000). As mentioned before, *Bifidobacterium* strains showed the highest growth rate on the oligosaccharide mixture produced by their own enzymes e.g. β-galactosidase (Rabiu et al. 2001). Another advantage could be that a different degree of polymerization and/or degree of branching might lead to less flatulent prebiotics (Cummings et al. 2001) and also a lower osmolarity can be obtained. Strategies to use and improve carbohydrate-modifying enzymes in prebiotic design will be discussed in the next paragraph.

Transglycosylation can be used to elongate carbohydrates. The main disadvantage of the retaining *Bifidobacterium* enzymes is that they possess also hydrolytic activity (besides transglycosylation activity). In most cases, the former is more predominant than the latter. For *Bifidobacterium* α-galactosidase we have found that under the right condition 69% of the hydrolyzed galactose units are used in the transglycosylation reaction with melibiose as substrate (Chapter 8; Van den Broek et al. 2005a). Usually, the balance between hydrolysis and transglycosylation is much less favorable. Different methods can be used to improve or optimize enzyme activities. Most of these methods are based on molecular genetic tools. Although not all of these methods have been applied for the improvement of *Bifidobacterium* carbohydrases, they can have potential to generate more efficient enzymes for industrial application.

Site-directed mutagenesis can be used to modify the enzyme activity. This can be achieved by mutations, insertion, or deletion of the nucleotides of the gene. For bifidobacteria only one attempt is reported. In this case the transglycosylation properties of α-galactosidase from *B. adolescentis* have been changed by site-directed mutagenesis (Chapter 8; Van den Broek et al. 2005a). Two mutated α-galactosidases showed a significant but small increase (3–6%) in transglycosylation activity in comparison to the wild type enzyme.

Rational prebiotic design. Fermentation studies of poly- and oligosaccharides and 'beneficial' bacteria were in most cases the basis for detection of prebiotic preparations. The *B*. *longum* genome sequence offers opportunities to rationalize prebiotic design, because a much clearer picture of the carbohydrate-degrading potential is now available. For instance, it appears that *B*. *longum* has an array of enzymes involved in the degradation of arabinoxylans, suggesting that these polysaccharides and/or oligosaccharides may be a suitable prebiotic. It should be realized that, although the completion of the *Bifidobacterium* genome sequence is a leap forward, there is still many aspects that should be approached with caution. (i) Polysaccharides (or oligosaccharides) can have many subtle structural details, which greatly influence their degradability by carbohydrases. For instance, cereal arabinoxylans can contain a large number of xylosyl residues that are doubly substituted by arabinose. Utilization of oligosaccharides containing such structural signatures strongly depends on having the appropriate enzymes. *B*. *adolescentis* is known to contain such an enzyme (Chapter 6; Van den Broek et al. 2005b; Van Laere et al. 1997; 1999b), but it is certainly not self-evident that all arabinofuranosidases can act on these side chains. Thus, available sequence information needs a thorough biochemical back-up at some stage. (ii) Feed-crossing by other microorganism may also play an important role for the availability of oligosaccharides in the GIT. (iii) It will be necessary to verify whether the extracellularly formed oligosaccharides can actually be internalized by *Bifidobacterium*. It is therefore important to establish a link between oligosaccharide structure and selectivity of the various transporter proteins, which will be a tremendous task. (iv) Last but not least, there is always the question whether a rationally designed *Bifidobacterium* prebiotic is a truly a selective substrate. An effective prebiotic use is dependent not only on the ability of bifidobacteria to utilize the compounds. In the human intestine it is proposed that more than 1000 bacterial species are present (Heselmans et al. 2004; Whitfield 2004), which can potentially all compete with bifidobacteria for these substrates.

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Chapter 8

Glycosyl hydrolases from *Bifidobacterium adolescentis* DSM20083

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ABSTRACT

It is claimed that bifidobacteria have several health-promoting effects. To increase the amount of bifidobacteria in the colon the concept of probiotics and/or prebiotics can be applied. *Bifidobacterium adolescentis* is one of the main species of bifidobacteria in the gastrointestinal tract of human adults. *B. adolescentis* is able to degrade a wide range of oligosaccharides and a number of glycosyl hydrolases have been characterized in detail. The hydrolytic activity of the glycosyl hydrolases from *B. adolescentis* toward prebiotics like arabinoxylan-oligosaccharides, isomalto-oligosaccharides, arabinogalactan, and sucrose-based oligosaccharides (raffinose, stachyose, and fructo-oligosaccharides) is reviewed. Alternatively, some of these glycosyl hydrolases are able to catalyze transglycosylation, which allows them to elongate oligosaccharides and to prepare potentially prebiotic oligosaccharides. Such oligosaccharides might be used to influence the microbial composition in the more distal parts of the colon. In nature, not all enzyme-substrate encounters are transglycosylating. So, the hydrolytic activity of the enzyme makes the oligosaccharide elongation less efficient as desired. Site-directed mutagenesis was applied to improve the transglycosylation reaction of the α-galactosidase from *B. adolescentis*. Furthermore, the application of the synthesized oligosaccharides in dairy products is discussed.

INTRODUCTION

The market for functional foods is rapidly growing, which is due to the fact that consumers are becoming more and more aware of the link between health, nutrition, and diet. Probiotics and/or prebiotics can be classified as functional food because it is claimed that they have health-promoting properties for the consumer (Hammes and Hertel 2002; Shah 2001; Stanton et al. 2001). A prebiotic is 'a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that have the potential to improve host health' (Gibson and Roberfroid 1995). It is generally accepted that mainly the growth of bacteria from the genera *Lactobacillus* or *Bifidobacterium* should be increased (Tomasik and Tomasik 2003). Examples of non-digestible oligosaccharides (NDOs), that can act as prebiotic, are fructo-oligosaccharides, α-galacto-oligosaccharides, β-galacto-oligosaccharides, and xylo-oligosaccharides (Gibson et al. 2000). It is assumed that bifidobacteria and lactic acid bacteria contain the required glycosyl hydrolases to convert the NDOs into fermentable sugars. This is corroborated by the genome sequence of *Bifidobacterium longum* (Schell et al. 2002) and *Lactobacillus lactis* (Kleerebezem et al. 2003), which indicate that an array of glycosyl hydrolases, glycosyl transferases, carbohydrate esterases, and carbohydrate binding modules is present. Especially *B. longum* contains a high number of these genes involved in carbohydrate metabolism (i.e. 5% in *B. longum* and 2.5% in *L. lactis*) indicating that they can utilize a wide range of carbohydrates.

Enzymatic hydrolysis of glycosidic bonds can be carried out with one of two stereochemical outcomes: net retention or net inversion of the anomeric configuration (Koshland 1953; Sinnot 1990). Retaining glycosyl hydrolases can transfer the non-reducing moiety of a carbohydrate donor molecule to a carbohydrate acceptor molecule instead of water, which yields elongated oligosaccharides with possibly new types of linkage. Inverting glycosyl hydrolases always use water as the acceptor molecule and are thus unable to catalyze elongation reactions.

In the gastrointestinal tract of human adults *B. adolescentis* is one of the predominant groups of *Bifidobacterium* sp. (Matsuki et al. 1999; Satokari et al. 2001). To understand the utilization of carbohydrates by *B. adolescentis*, a more detailed study of their glycosyl hydrolases is required. Therefore, in the first part we have embarked on investigating the substrate specificity of glycosyl hydrolases from *B. adolescentis* DSM20083, in particular to understand why certain oligosaccharides can have a prebiotic function. Recently, it was found that *Bifidobacterium* sp. had

the highest growth rate on β-galacto-oligosaccharides mixtures produced from lactose by its 'own' β-galactosidases (Rabiu et al. 2001). This underlines the potential of using retaining enzymes from bifidobacteria for synthesis of prebiotic oligosaccharides by transglycosylation. In the second part, we report on site-directed mutagenesis to increase the transglycosylation activity of the α-galactosidase from *B. adolescentis*.

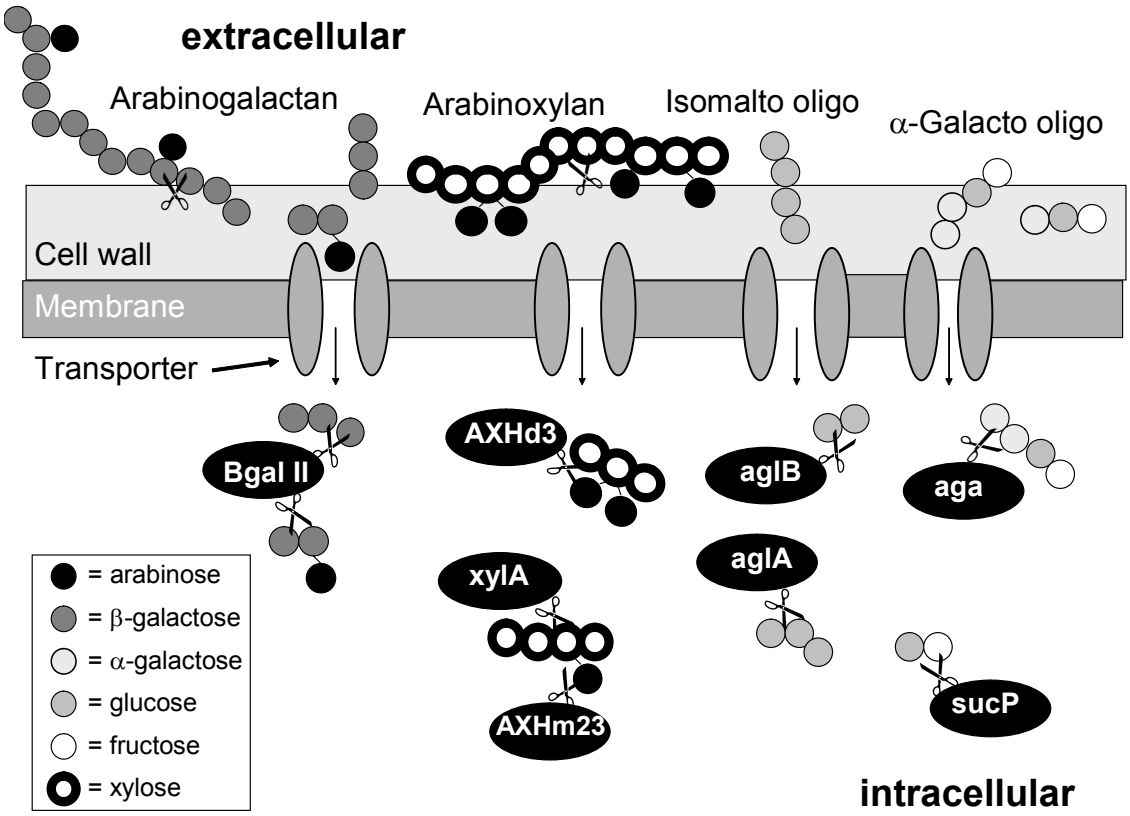

Figure 1: Overview of isolated and/or cloned glycosyl hydrolases from *Bifidobacterium adolescentis* DSM20083 able to utilize arabinoxylan-oligosaccharides, isomalto-oligosaccharides, α-galacto-oligosaccharides, and arabinogalactan. aga is α-galactosidase; aglA and aglB are α-glucosidases; sucP is sucrose phosphorylase; AXHd3 and AXHm23 are arabinoxylan arabinofuranohydrolases D3 and m23, respectively; xylA is a putative xylanase; and bgal II is β-galactosidase.

GLYCOSYL HYDROLASES FROM *BIFIDOBACTERIUM ADOLESCENTIS*

Carbohydrates play an important role in the gastrointestinal tract of humans. Besides their direct physiological effect they also affect the gut ecosystem, which significantly contribute to the well being of humans (Falk et al. 1998). To influence the microbiota, especially to increase the number of bifidobacteria, more knowledge is needed what kind of oligo- and polysaccharides can be used. Identification and biochemical characterization of glycosyl hydrolases from bifidobacteria will give more insight in this. Although, there is a strong competition in the colon between the different microorganisms for the various carbon sources, and also other inhabitants may, besides bifidobacteria, proliferate that were not detected to date. In Figure 1 is a cartoon that gives an overview of some well described glycosyl hydrolases of *B. adolescentis* DSM20083 (Chapter 2; 3; 4; 6; Hinz et al. 2004; Van den Broek et al. 1999; 2003; 2004; 2005; Van Laere et al. 1997; 1999b; 2000a). Here, we discuss the possible role of these glycosyl hydrolases involved in the utilization of prebiotics by *B. adolescentis*.

Arabinoxylan. It is known that arabinoxylan-oligosaccharides (AXOS) can be fermented by *B. longum* and *B. adolescentis* (Van Laere et al. 2000b). Van Laere et al. (1997; 1999b) characterized two different arabinofuranosidases from *B. adolescentis* able to release arabinose residues from arabinoxylan. These enzymes were named AXHd3, which hydrolyzed only C3-linked arabinosyl residues from double-substituted xylosyl residues, and AXHm23, which released only arabinosyl residues that were C2 or C3 linked of single-substituted xylosyl residues. Together with β-xylosidase these two enzymes were able to degrade AXOS completely.

In chapter 6 the cloning of AXHd3 was described and another gene preceding this sequence was also identified (Van den Broek et al. 2005). This gene (*xylA*) showed high homology with a putative endo-xylanase from *Bacillus halodurans*. The enzyme could be responsible for degradation of arabinoxylan into oligomers. However, the *xylA* gene did not contain a sequence for a secretion signal peptide. It remains to be determined if this is a true endo-xylanase or a glucanase, because this enzyme is classified into glycoside hydrolase family (GH-family) 8 which contains mainly glucanases. The cloning and characterization of this enzyme is now in progress.

The presence of the above-mentioned enzymes shows why *B. adolescentis* is able to grow on AXOS. *B. longum* is also able to ferment this substrate and its genome sequence revealed thirteen putative genes involved in arabinoxylan degradation (Schell et al. 2002). In contrast

preliminary results of the genome sequence of *B*. *breve* UCC2003 showed the absence of arabinoxylan degrading enzymes (Van Sinderen 2004), indicating that AXOS has some selectivity within the genus *Bifidobacterium*.

Isomalto-oligosaccharides. One of the prebiotic products on the Japanese market is composed of isomalto-oligosaccharides (Crittenden and Playne 1996). These oligosaccharides contain glucose units that are α-(1,6)-linked. Two α-glucosidases genes have been cloned from *B*. *adolescentis* (Chapter 3; Van den Broek et al. 2003). AglB (EC 3.2.1.20) was able to degrade maltose (α-(1,4)-linkage) and isomaltose (α-(1,6)-linkage), but not isomaltotriose. AglA (EC 3.2.1.10) showed no activity towards maltose but showed high activity toward isomaltose and isomaltotriose. AlgA and not AglB could play a role in the utilization of isomalto-oligosaccharides by *B*. *adolescentis*.

Sucrose-based oligosaccharides. α-Galacto-oligosaccharides like raffinose and stachyose are assumed to promote bifidobacterial growth in the colon (Gibson et al. 2000). *B*. *adolescentis* contains an α-galactosidase (Chapter 2; Leder et al. 1999; Van den Broek et al. 1999; Van Laere et al. 1999a), which is able to split off the galactosyl units from these oligosaccharides, resulting in the formation of galactose and sucrose.

Sucrose can be converted by sucrose phosphorylase to D-fructose and glucose 1-phosphate. This reaction is energetically advantageous, because it is an alternative for the ATP-requiring phosphorylation of glucose by hexokinase. Therefore, sucrose phosphorylase might play an important role in the fermentation pathway of sucrose obtained after hydrolysis of raffinose and stachyose. Sucrose phosphorylase genes have been cloned from *B*. *adolescentis* (Chapter 4; Van den Broek et al. 2004), *B*. *lactis* (Trindade et al. 2003), and *B*. *longum* (Kim et al. 2003).

Another well studied prebiotic belongs to the group of fructo-oligosaccharides (Gibson et al. 2000). Some of these oligosaccharides contain a terminal sucrose unit (GF-type), such as in inulin. *Bifidobacterium* sp. produce a β-fructofuranosidase (Muramatsu et al. 19992) able to split off fructosyl units from inulin, which finally results in the release of sucrose.

Arabinogalactan. *B*. *adolescentis* and other *Bifidobacterium* sp. are able to degrade arabinogalactan-oligosaccharides (Van Laere et al. 2000b). Recently, we have cloned the β-galactosidase from *B*. *adolescentis* (Hinz et al. 2004), which was only induced by β-galacto-oligosaccharides with a higher degree of polymerization (>2; Van Laere et al. 1999a). Whereas most β-galactosidases from *Bifidobacterium* sp. have a preference for lactose, this enzyme showed preference for β-(1,4)-galactosides, such as in arabinogalactan-oligosaccharides derived from potato galactan. The genome sequence of *B*. *longum* reveals an endo-galactanase, which is probably

membrane-anchored. We have expressed this enzyme and demonstrated that it can degrade arabinogalactan. It remains to be established whether *B*. *adolescentis* produces such an enzyme. It is also possible that *B*. *longum* produces arabinogalactan-oligosaccharides, which can be utilized by *B*. *adolescentis* in vivo. The presence of a β-galactosidase with preference for β-(1,4)-linkages together with an endo-galactanase may account for a rather complete system for the utilization of arabinogalactan in bifidobacteria.

IMPROVEMENT OF THE TRANSGLYCOSYLATION ACTIVITY OF α-GALACTOSIDASE FROM *BIFIDOBACTERIUM ADOLESCENTIS*

As mentioned above the use of *Bifidobacterium* enzymes for oligosaccharide synthesis can be advantageous. It is also discussed that elongated oligosaccharides may exert an enhanced prebiotic effect in the more distal colonic region, where most disorders of the gut are encountered (Gibson et al. 2000). Retaining glycosyl hydrolases (Table 1) offer the opportunity to make such longer oligosaccharides; however, there is always a competition between transglycosylation and hydrolysis. Depending of the enzyme hydrolysis often exceeds transglycosylation. Experiments in our laboratory have shown that α-galactosidase from *B*. *adolescentis* is a rather efficient transglycosylase in comparison with the other retaining glycosyl hydrolyses (Chapter 2, 3, 4; Hinz et al. 2004; Van den Broek et al. 1999; 2003; 2004; Van Laere et al. 1999a). Therefore, α-galactosidase was chosen for further improvement of its transglycosylation activity

Site-directed mutagenesis of α-galactosidase from *Bifidobacterium adolescentis*. The literature shows that transglycosylation can be improved by site-directed mutagenesis of the amino acids in the vicinity of the catalytic amino acids (Hansson and Adlercreutz 2000; Kuriki et al. 1996; Matsui et al. 1994). However, the α-galactosidase of *B*. *adolescentis* belongs to GH-family 36 of which no 3D structure is currently available, and information on its catalytic residues is lacking. On the other hand, GH-family 36 enzymes, containing primarily α-galactosidases from prokaryotic origin, have a strong relationship with GH-family 27 enzymes, which contain mainly α-galactosidases from eukaryotic origin. Hart et al. (2000) and Ly et al. (2000) identified the catalytic nucleophile in the main α-galactosidase from *Phanerochaete chrysosporium* and from green coffee bean, respectively,

both belonging to GH-family 27. The position of the catalytic nucleophile was confirmed by work from Garman and Barboczi (2004), who solved the 3D structure of human α-galactosidase (GH family 27). To identify the catalytic nucleophile of the α-galactosidase from *B*. *adolescentis* an amino acid alignment was performed with the highly conserved consensus region of all GH-family 27 enzymes containing the catalytic nucleophile and with the corresponding conserved consensus region of all GH-family 36 enzymes. Based on the alignment, we propose that D496 in YIKW**D** is the catalytic nucleophile in the α-galactosidase of *B*. *adolescentis*.

TABLE 1: Cloned genes from *Bifidobacterium adolescentis* DSM20083

Enzyme	Accession number	Gene	GH-family[a]	Retaining/ Inverting
Arabinoxylan arabinofuranohydrolase-D3	AY233379	*axhD3*	43	Inverting
α-Galactosidase	AF124596	*aga*	36	Retaining
β-Galactosidase	AY359872	*bgal* II	42	Retaining
α-Glucosidase	AF358444	*aglA*	13	Retaining
α-Glucosidase	AF411186	*aglB*	13	Retaining
Sucrose phosphorylase	AF543301	*sucP*	13	Retaining

[a] GH-family is glycoside hydrolase family

In vitro site-directed mutagenesis was applied to alter a number of amino acids of the α-galactosidase in the vicinity of the putative catalytic nucleophile. The gene coding for α-galactosidase from *B*. *adolescentis* (Chapter 2; Van den Broek et al. 1999) was amplified with *Pfu* Turbo polymerase (Stratagene) and the primers GALFOR and GALREV (Table 2). The primer GALFOR contained an *Xba*I site and the primer GALREV a *Hind*III site. After amplification the gene was digested with *Xba*I and *Hind*III and ligated into the *p*Bluescript vector (Promega), which was previously digested with these two restriction enzymes. Site-directed mutagenesis of the α-galactosidase was performed according to the instructions of the supplier of the Quickchange site-directed mutagenesis kit (Stratagene). Three different mutants were created H497M, K499R, and Y500L by changing the nucleotide sequence accordingly (Table 2). Plasmids were isolated using

the High Pure PCR Product Purification Kit (Boehringer) and were sequenced with an automated DNA-sequencer 373 (Applied Biosystems).

TABLE 2: Primers used for amplification and site-directed mutagenesis of the α-galactosidase from *Bifidobacterium adolescentis* DSM20083. For site-directed mutagenesis both the forward and reverse primer are indicated. Altered nucleotides are indicated in bold face type, the restrictions site containing the silent mutation[a] is underlined

Primer	5′ → 3′
GALFOR	gcgctctagagcaatgacgctcattca
GALREV	cgcgaagctttactcagatgcggacta
H497M	cggcatcgat**t**acatcaaatgggat**atg**aacaaatacgtcaccg
	cggtgacgtatttgtt**cat**atcccatttgatgta**a**tcgatgccg
K499R	cggcatcgat**t**acatcaaatgggatcacaac**cgc**tacgtcaccg
	cggtgacgta**gcg**gttgtgatcccatttgatgta**a**tcgatgccg
Y500L	ggatcacaacaaa**ctc**gtcaccgaagcggtgtcg**ccgcgg**accgg
	ccggt**c**cgcggcgacaccgcttcggtgac**gag**tttgttgtgatcc

[a] Silent mutations were created by changing the nucleotide sequence without changing the amino acid sequence, to check by restrictions analysis (*Cla*I and *Sac*II) if the desired mutant was obtained

In a preliminary study it was observed that the degree of transglycosylation was increased at a higher pH. Based on these results the mutations H497M and K499R were made to influence the charge in the catalytic centre. Histidine contains a basic side-chain, whereas methionine has a neutral side-chain. The difference between lysine and arginine lies in the p*K* values of their basic side-chains, 10.5 and 12.5, respectively. The mutant Y500L was based on results of Matsui et al. (1994). They hypothesized that, in the case of α-amylase, the aromatic ring of tyrosine was involved in the binding of oligomeric substrates through a so-called "stacking interaction", whereas the hydroxyl group of tyrosine played a role in the fixation of the catalytic water molecule. Mutation of tyrosine to a non-aromatic residue (leucine) could lead to less precise positioning of the water molecule and thereby favoring transglycosylation over hydrolysis. The different mutated α-

galactosidase genes were cloned into *E. coli*, and the obtained enzymes were tested for their hydrolytic and transglycosylation activity.

The mutant enzymes were active as judged from their activity toward *p*-nitrophenyl-α-D-galactopyranoside (Chapter 2; Van den Broek et al. 1999). Wild type and mutant enzymes, purified from the supernatant with aid of a Q-sepharose anion exchange column (Amersham Biosciences), were incubated with melibiose at pH 8 and the degree of transglycosylation was calculated from the amount of glucose minus galactose, divided by the amount of glucose determined by HPAEC (Chapter 2; Van den Broek et al. 1999). Y500L and H497M mutants showed a significantly higher degree of transglycosylation, (72 and 75%, respectively) than the wild type enzyme (69%), whereas the mutant K499R showed a lower degree of transglycosylation (67%; Figure 2). These results showed that the transglycosylation activity could be increased, which is a first step towards a more efficient production of elongated oligosaccharides. Our results indicated that subtle changes around the catalytic nucleophile could influence the likelihood of transglycosylation. However, the effect of these mutations is more difficult to explain. More research is needed to elucidate the precise mechanism by which transglycosylation can be influenced.

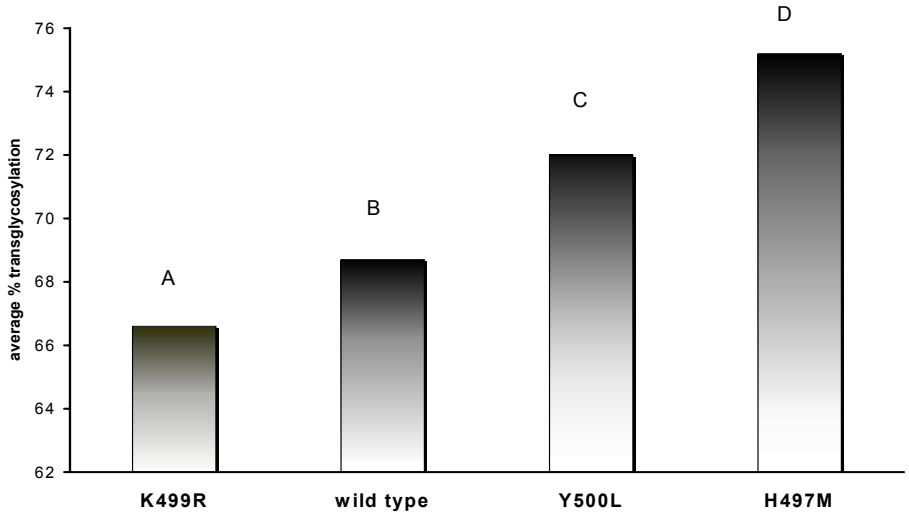

Figure 2: Average % transglycosylation activity of wild type and mutated α-galactosidases from *Bifidobacterium adolescentis* DSM20083 (n=5) incubated with melibiose as substrate at pH 8 for 16 h. A, B, C, and D represent significant difference between the mutated enzymes. Variation analysis (Oneway Anova; $p<0.05$) and a post-hoc test (Duncan's multiple comparison; $\alpha<0.05$) were used to determine significant differences between the transglyscosylation activities.

APPLICATION OF TRANSGLYCOSYLATION PRODUCTS IN DAIRY SCIENCE

Prebiotics can be applied to target strains naturally present in the gut that have particular health benefits (Rastall and Gibson 2002). Transglycosylation products can be used as prebiotics or in synbiotics to achieve this. These products are already marketed as dairy products in Europe and Japan (Young 1998). It is also possible to use the transglycosylation activity of enzymes from probiotic microorganisms to synthesize prebiotics in food products. Lamoureux et al. (2002) used mixed cultures of bifidobacteria in the preparation of yoghurts. These bifidobacteria contained β-galactosidase activities that were responsible for the extracellular production of oligosaccharides with a polymerization degree of 3 from lactose. Important prerequisites for production of elongated oligosaccharides in the product are the extracellular location of the β-galactosidases and the ability of sufficiently high concentration of donor molecules (i.e. lactose). However, most β-galactosidases are not secreted by bifidobacteria. The β-galactosidase genes from *B*. *longum* NCC2705 (Schell et al. 2002) do not have a sequence encoding a signal peptide, and only one of the three β-galactosidases from *B*. *bifidum* contained a signal peptide (Møller et al. 2001). It was not investigated, which of these β-galactosidases were responsible for the transglycosylation products, although *B*. *infantis* showed the highest production of oligosaccharides.

In our studies we have investigated the transglycosylation activity of an α-galactosidase from *B*. *adolescentis*. In order to produce oligosaccharides from raffinose and/or stachyose in for example soymilk this enzyme should be secreted by *B*. *adolescentis*. However, this enzyme does not contain a signal peptide and the enzyme is located in the cell. The structure of the trisaccharide and tetrasaccharide synthesized from melibiose by the wild type enzyme were elucidated by NMR (α-D-Gal*p*(1,6)-α-D-Gal*p*(1,6)-D-Glc*p* and α-D-Gal*p*(1,6)-α-D-Gal*p*(1,6)-α-D-Gal*p*(1,6)-D-Glc*p*, respectively). The structure of these oligomers indicated that selective transglycosylation took place at the C6-hydroxyl group. Contrary to the example provided by Lamoureux et al. (2002), these oligosaccharides should be added as ingredient to a probiotic strain to obtain a synbiotic, and are not produced in the product itself.

The genome sequence of *B*. *longum* revealed that most glycosyl hydrolases contain no signal peptide and are thus not secreted (Schell et al. 2002). This makes bifidobacteria in most cases less suitable for the development of synbiotics, in which they synthesize their own prebiotics. Another

drawback could be the low concentration of donor molecules in the product, which limits the synthesis of elongated oligosaccharides. Therefore, it seems more appropriate to add prebiotics to probiotic strains, instead of making them in a product. In order to reduce productions costs the transglycosylation activity of glycosyl hydrolases should be increased. Site-directed mutagenesis can be one way to achieve this as indicated for the α-galactosidase from *B*. *adolescentis*. Therefore, more research is needed to be able to influence the transglycosylation activity of glycosyl hydrolases for a higher yield of elongated oligosaccharides. The next step is to investigate if these prebiotics and/or synbiotics have a real contribution to the well being of humans.

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Chapter 9

Concluding remarks

The research described in this thesis consist of two parts (i) analysis of the carbohydrate modifying enzyme toolbox of *Bifidobacterium adolescentis* and (ii) to study the possibilities to synthesize tailor-made oligosaccharides using the glycosyl hydrolases from *B. adolescentis*. Knowledge about the enzyme toolbox can give more information what kind of poly- and oligosaccharides can be degraded and utilized by *B. adolescentis*. The second objective was to produce enzymatic synthesized oligosaccharides, which can be used as prebiotic substrates for bifidobacteria. In this chapter the main concluding remarks are given of this study.

Enzyme toolbox of *Bifidobacterium adolescentis*. The first aim of this thesis was to study the carbohydrate modifying enzyme toolbox of *B. adolescentis*. A genomic library of *B. adolescentis* DSM20083 was constructed and screened for glycosyl hydrolases, using different 4-methyl-umbelliferyl glycosides as substrate. Three different glycosyl hydrolases were identified; an α-galactosidase (chapter 2) and two α-glucosidases (chapter 3). It is known that *B. adolescentis* contains a whole range of glycosyl hydrolases active towards 4-methyl-umbelliferyl glycosides, although only three genes were identified in this study. The fact that not more enzymes were found can be due to (i) that not all these enzymes are expressed, although the genomic DNA used for library construction was only partial digested, that resulted in a whole range of different DNA-fragments or (ii) that these enzymes are not active in *Escherichia coli*. For screening of other glycosyl hydrolases a genomic library can be constructed using different restriction enzymes to digest the DNA from *B. adolescentis*. Another option is to design primers for PCR from known glycosyl hydrolase genes from other *Bifidobacterium* sp. to amplify the genes of interest. A pitfall can be that not always the same glycosyl hydrolases are present in *Bifidobacterium* sp. or that another nucleotide sequence is used.

Cloning and expression of the glycosyl hydrolases was also performed to obtain a higher yield of the enzymes. The yield of the recombinant α-galactosidase (chapter 2) was 100 times higher as the native α-galactosidase and only s single purification step was needed. The cloning of arabinofuranohydrolase-D3 (AXHd3) resulted in a much higher production of this enzyme (chapter 6) in comparison with the native enzyme. Therefore, a better biochemical characterization was possible, because the amount of enzyme was not limited.

The gene sequence of AXHd3 showed that the gene had high homology with putative xylosidases. However, the biochemical characterization of AXHd3 (chapter 6) showed that the enzyme releases arabinose units from xylose moieties and might play a role in the degradation of arabinoxylan-oligosaccharides. It is possible that the putative xylosidase, with high homology for

the *axhdD3* gene, show also arabinose releasing activities instead of releasing xylose moieties. In this study two α-glucosidase genes were identified (chapter 3). Biochemical characterization showed that both enzymes had different substrate specificity. AglB showed a more broad substrate specificity, whereas AglA on the other hand showed a more narrow substrate specificity. AglA posses high hydrolytic activity towards isomaltose and isomaltotriose and might play an important role in the degradation of isomalto-oligosaccharides, which can be used as prebiotic. From the sequence alone it cannot be predicted what the precise mode of action is. Therefore, a biochemical characterization of the enzymes is essential to determine their mode of action.

In the glycolysis pathway glucose is converted by hexokinase to glucose 1-phosphate. The presence of sucrose phosphorylase can be advantageous for bifidobacteria, because it is bypassing this ATP-requiring step of the hexokinase reaction to phosphorylate glucose, derived from sucrose (chapter 4). α-Galactosidase can play an important role in the production of sucrose for sucrose phosphorylase by degrading raffinose and stachyose. In addition, fructofuranosidases are able to produce sucrose from fructo-oligosaccharides, which have a glucose-unit at their non-reducing end. Both α-galacto-oligosaccharides (raffinose and stachyose) and fructo-oligosaccharides are used as prebiotic.

The 3D structure of a sucrose phosphorylase is presented (chapter 5). This is the first solved 3D structure of a phosphate-dependent enzyme from glycoside hydrolase family 13. For further investigation of the reaction pathway co-crystallization experiments have to be set up with e.g. sucrose and phosphate. Also mutants can be created to catch sucrose at the active site, which can give information about the possible binding site for the substrate.

The biochemical characterization of the enzymes showed that most enzymes prefer dimers or oligosaccharides (chapter 7 and 8). In addition the genome sequence of *B*. *longum* showed that most glycosyl hydrolases are intracellularly located and a high number of putative sugar transporters were identified. This indicates that bifidobacteria can utilize oligosaccharides very well. Only a limited number of endo-enzymes were identified in the genome of *B*. l*ongum*. Therefore, other microorganisms may provide bifidobacteria with oligosaccharides, which can be transported into the cell where they can be utilized further. In this way, feed crossing might be important in the colon for the growth of bifidobacteria. Another important aspect is that besides feed crossing, there will be a strong competition between other microorganisms for the available substrates. The presence of certain enzymes may be beneficial for bifidobacteria to degrade oligosaccharides for their own use e.g. β-galactosidases for the degradation of lactose. Another

aspect is that the genome sequence of *B*. *longum* and *B*. *breve* showed that there is some selectivity within the genus *Bifidobacterium* due to the absence or presence of certain enzyme (e.g. arabinoxylan-degrading enzymes). To obtain more knowledge about prebiotic function and the growth of bifidobacteria it is interesting to study the different sugar transporters that specific transport oligosaccharides into the cells of bifidobacteria.

Transglycosylation activity of glycosyl hydrolases from *B. adolescentis*. The second aim was to study the possibilities to synthesize tailor-made oligosaccharides using the glycosyl hydrolases from *B*. *adolescentis*. The reason for this is that (i) longer oligosaccharides may further enter the colon, where most disorders take place, and (ii) the likelihood that *Bifidobacterium* glycosyl hydrolases can hydrolyze these newly synthesized oligosaccharides, because all the tools for their utilization are available. This way the oligosaccharides might have the potential to act as preferential prebiotic. Only retaining enzymes have the possibility to show transglycosylation activity. Different enzymes from *B*. *adolescentis* showed transglycosylation activity like α-galactosidase (chapter 2), α-glucosidases (chapter 3), and sucrose phosphorylase (chapter 4).

The retaining enzymes produced a wide range of oligosaccharides. α-Galactosidase synthesized new α-galacto-oligosaccharides from melibiose and stachyose. The two α-glucosidases showed different transglycosylation properties. AglA was able to synthesize oligosaccharides from trehalose and sucrose. AglB formed oligosaccharides from sucrose, maltose, and melizitose. Both enzymes produced different products and had a different ratio of synthesized oligosaccharides with sucrose as substrate. This is due to the fact that both enzymes have different substrate specificity (preference for different linkages) for hydrolysis. Sucrose phosphorylase was able to use a broad range of monomeric sugars as acceptor in the transglycosylation reaction, while α-glucose 1-phosphate served as donor. D- and L-arabinose, D- and L-arbitol, and xylitol showed the highest production of transglycosylation products. A new synthesized non-reducing dimer, α-Glc*p*(1→1)β-Ara*f*, was formed by sucrose phosphorylase using D-arabinose as acceptor. The investigated dimer and trimers were not suitable as acceptor, which is agreement with the 3D structure of sucrose phosphorylase (chapter 5), which showed that the B´ domain disfavors oligosaccharides binding. The products formed by sucrose phosphorylase or synthesized by α-galactosidase and α-glucosidases should be further elucidated.

It was shown that the retaining enzymes were able to produce new dimers or elongated oligosaccharides. The new synthesized product should be tested for their bifidogenic and prebiotic properties. The simplest test is to investigate if bifidobacteria can grow on these substrates. The

next step is to grow bifidobacteria on these carbon sources in combination with other gut microorganisms and to determine if the growth of bifidobacteria is stimulated. The last step is to determine if there is any prebiotic effect in human trials.

To apply enzymatic synthesized oligosaccharides as prebiotic a high production is needed. Therefore, it is important to increase the enzyme activity of retaining glycosyl hydrolases in favor of their transglycosylation activity instead of their hydrolytic activity. It was shown that it is possible to increase the transglycosylation activity of α-galactosidase by site-directed mutagenesis (chapter 8). Another approach to increase the synthesizing properties can be directed evolution.

Conclusion. The biochemical characterization of the glycosyl hydrolases from bifidobacteria gives additional information why some oligosaccharides act as prebiotic. In addition the genomes sequences of these organisms give valuable information what kind of substrates can be used as carbon source. Another use of the retaining glycosyl hydrolases is that these can be applied for synthesizing new and/or elongated oligosaccharides, which might act as potential prebiotics. Although information about the glycosyl hydrolases from bifidobacteria is still limited, it gives useful information why these microorganisms are so well adapted in the colon of humans.

SUMMARY

Nowadays, there is increasing interest in (or market for) ingredients, which positively influence the intestinal microbiota through the diet; e.g. probiotics and/or prebiotics. It is claimed that microorganisms belonging to the genera *Bifidobacterium* and *Lactobacillus* have health promoting properties. The aim of the research was (i) to identify the carbohydrate modifying enzyme toolbox of *Bifidobacterium adolescentis,* one of the main *Bifidobacterium* sp. in the colon of human adults. Bifidobacteria contain a high number of genes involved in the carbohydrate metabolism and information about their carbohydrases will give more information on what kind of poly- and oligosaccharides can be degraded. The second objective was (ii) to study the possibilities to synthesize tailor made oligosaccharides using the glycosyl hydrolases from *B*. *adolescentis*. This way it is possible to produce oligosaccharides, which might be used as preferred prebiotic.

In the general introduction (chapter 1) functional food ingredients like probiotic, prebiotic, and synbiotic are described. Also a general background is given about carbohydrases and bifidobacteria.

Chapter 2 describes the construction of a genomic library of *B*. *adolescentis* DSM20083 in *Escherichia coli*. The genomic library was screened for glycosyl hydrolases and a gene enoding a α-galactosidase (*aga*) was isolated. The identified open reading frame showed high similarity and identity with bacterial α-galactosidases, which belong to family 36 of the glycoside hydrolases. For the purification of the recombinant enzyme form the medium a single chromatography step was sufficient. The yield of the recombinant α-galactosidase was 100 times higher than from *B*. *adolescentis* itself. In addition to hydrolytic enzyme activity the α-galactosidase showed transglycosylation activity and can be used for the production of α-galacto-oligosaccarides, which might be used as prebiotic.

Screening of the genomic library (chapter 3) resulted also in the identification of two α-glucosidase encoding genes (*aglA* and *aglB*). Both α-glucosidases belong to family 13 of the glycoside hydrolases. AglA (EC 3.2.1.10) and AglB (EC3.2.1.20) expressed in *E*. *coli*, showed high hydrolytic activity towards isomaltose and *p*np-α-glucoside. The K_m for *p*np-α-glucoside was 1.05 and 0.47 mM and the V_{max} was 228 and 113 U mg^{-1} for AglA and AglB, respectively. Using *p*np-α-glucoside as substrate, the pH optimum for AglA was 6.6 and the temperature optimum was 37 ^oC. For AglB, values of pH 6.8 and 47 ^oC were found. AglA also showed high hydrolytic activity towards isomaltotriose, and, to a lesser extent, towards trehalose. AglB has a high preference for

maltose and less activity towards sucrose; minor activity was observed towards melizitose, low molecular weight dextrins, maltitol, and maltotriose. The α-glucosidases were tested for their transglycosylation activity. AglA was able to synthesize oligosaccharides from trehalose and sucrose. AglB formed oligosaccharides from sucrose, maltose, and melizitose.

Clones of the genomic library of *B. adolescentis* were grown in minimal medium with sucrose as sole carbon source (chapter 4). An enzymatic fructose dehydrogenase assay was used to identify sucrose degrading enzymes. Plasmids were isolated from the positive colonies and sequence analysis revealed that two types of inserts were present, which only differed with respect to the orientation in the plasmid. An open reading frame of 1,515 nucleotides with high homology for sucrose phosphorylases was detected on these inserts. The gene was designated *sucP* and encodes a protein of 56,189 Da. *SucP* was heterologously expressed in *E. coli*, purified, and characterized. The molecular mass of SucP was 58 kDa as estimated by SDS-PAGE while with gel permeation chromatography 129 kDa was found, suggesting that the native enzyme is a dimer. The enzyme showed high activity towards sucrose and to a lower extent towards α-glucose 1-phosphate. The transglucosylation properties were investigated using a broad range of monomeric sugars as acceptor substrate for the recombinant enzyme, while α-glucose 1-phosphate served as donor. D- and L-arabinose, D- and L-arabitol, and xylitol showed the highest production of transglucosylation products. The investigated disaccharides and trisaccharides were not suitable as acceptor. The structure of the transglucosylation product obtained with D-arabinose as acceptor was elucidated by NMR. The structure of the synthesized non-reducing dimer was α-Glc*p*(1→1)β-Ara*f*.

In chapter 5 the crystal structure of sucrose phosphorylase is presented, which is refined at 1.77 Å resolution. It represents the first 3D structure of a sucrose phosphorylase and is the first structure of a phosphate-dependent enzyme from the glycoside hydrolase family 13. The structure is composed of the four domains, A, B, B´, and C. Domain A comprises the $(\beta/\alpha)_8$-barrel common to family 13. The catalytic active-site residues (Asp192 and Glu232) are located at the tips of β-sheets 4 and 5 in the $(\beta/\alpha)_8$-barrel, as required for family 13 members. The topology of the B´ domain disfavors oligosaccharide binding and reduces the size of the substrate access channel compared to other family 13 members, which are not phosphate dependent, underlining the role of this domain in modulating the function of these enzymes. It is remarkable that the fold of the C domain is not observed in any other known hydrolases of family 13. Sucrose phosphorylase was found as a homodimer in the crystal, and a dimer contact surface area of 960 Å per monomer was calculated. The majority of the interactions are confined to the two B domains, but interactions between the

loop 8 regions of the two barrels are also observed. This results in a large cavity in the dimer, including the entrance to the two active sites.

Arabinoxylan arabinofuranohydrolase-D3 (AXHd3) from *B. adolescentis* releases only C3-linked arabinose residues from double-substituted xylose residues. The genomic library was screened for the presence of the *axhD3* gene (chapter 6). Two plasmids were identified harboring part of the *axhD3* gene. The nucleotide sequences were combined and three open reading frames (ORFs) were found. The first ORF showed high homology with xylanases belonging to family 8 of the glycoside hydrolases and this gene was designated *xylA*. The second ORF was the *axhD3* gene belonging to glycoside hydrolase family 43. The third (partial) ORF coded for a putative carboxylesterase. Only the *axhD3* gene was cloned and expressed in *E. coli*. Different substrates were tested for biochemical characterization of the recombinant AXHd3. The enzyme showed the highest activity toward wheat arabinoxylan oligosaccharides. In addition, β-xylanase from *Trichoderma* sp. was able to degrade soluble wheat arabinoxylan polymer to a higher extent, after pretreatment with AXHd3. Arabinoxylan oligosaccharides incubated with a combination of AXHd3 and an α-L-arabinofuranosidase from *Aspergillus niger* did not result in a higher maximal release of arabinose than incubated with these enzymes separately.

Chapter 7 presents a review of the carbohydrate-modifying enzymes of bifidobacteria. Recently, the genome sequence of *Bifidobacterium longum* was completed and it was observed that more than 8% of the annotated genes were involved in carbohydrate metabolism. Besides, more sequence data of individual carbohydrases from other *Bifidobacterium* sp. became available. Together, these data allow us to pinpoint carbohydrate-modifying enzymes, what kind of poly- and oligosaccharides they can utilize, and enabling a much more directed selection of carbohydrates for prebiotic use. The carbohydrases of bifidobacteria can also have potential for tailoring more complex or longer carbohydrate structures, at least if they are capable of catalyzing transglycosylation reactions. It is thought that such oligosaccharides might be used to influence the microbial composition of the more distal parts of the colon. The advantage of using *Bifidobacterium*'s own carbohydrases for this purpose is the increased likelihood that this organism can utilize the newly synthesized products. Approaches to use and improve carbohydrate-modifying enzymes in prebiotic design are discussed.

In chapter 8 an overview is given of a number of glycosyl hydrolases from *B. adolescentis* that have been characterized in detail as presented in previous chapters (2, 3, 4, 6). The hydrolytic activity of the glycosyl hydrolases from *B. adolescentis* toward prebiotics like arabinoxylan-oligosaccharides, isomalto-oligosaccharides, arabinogalactan, and sucrose-based oligosaccharides

(raffinose, stachyose, and fructo-oligosaccharides) is reviewed. In addition, data is presented how site-directed mutagenesis was applied to improve the transglycosylation reaction of the α-galactosidase from *B. adolescentis* described in chapter 2. Furthermore, the application of the synthesized oligosaccharides in dairy products is discussed

Finally, in chapter 9 the concluding remarks are given of this study.

SAMENVATTING

Er is een toenemende interesse en vraag, ook vanuit de levensmiddelenindustrie, om de darm flora op een positieve manier te beïnvloeden door het toevoegen van probiotica en/of prebiotica aan het dieet. Er zijn claims dat micro-organismen die behoren tot de genera *Bifidobacterium* and *Lactobacillus* gezondheidsbevorderende eigenschappen hebben voor de gastheer (mens en dier). Het doel van dit onderzoek was tweeledig. De eerste doelstelling was het identificeren van koolhydraatmodificerende enzymen van *Bifidobacterium adolescentis* DSM20083. Dit micro-organisme is een van de meest voorkomende bifidobacteriën in de dikke darm van volwassenen. Bifidobacteriën hebben een groot aantal genen die betrokken zijn bij koolhydraatmetabolisme. Informatie over deze koolhydraatsplitsende enzymen kan leiden tot meer inzicht in welke poly- and oligosacchariden afgebroken kunnen worden. De tweede doelstelling was het bestuderen van de mogelijkheden om 'op maat' gemaakte oligosacchariden te synthetiseren met behulp van *B. adolescentis* eigen glycosylhydrolases. Dit is een manier om oligosacchariden te produceren die eventueel gebruikt kunnen worden als preferente prebiotica.

In de algemene introductie (hoofdstuk 1) worden de omschrijvingen gegeven van functionele levensmiddelen, probiotica en prebiotica. Ook wordt er achtergrond informatie gegeven over koolhydraatsplitsende enzymen en over bifidobacteriën.

Hoofdstuk 2 beschrijft de constructie van een genomische bank van *B. adolescentis* DSM20083 in *Escherichia coli*. De genomische bank werd getest op aanwezigheid van glycosylhydrolases en een gen coderend voor α-galactosidase werd geïsoleerd. De geïdentificeerd open reading frame had een hoge mate van identiteit en overeenkomst met α-galactosidases van bacteriën, welke behoren tot de familie 36 van de glycosylhydrolases. Met slechts een enkele chromatografiestap was het mogelijk om α-galactosidase te zuiveren uit het cultuurfiltraat. De opbrengst van α-galactosidase was 100 maal hoger in vergelijking met het enzym gezuiverd uit *B. adolescentis*. Naast hydrolyse activiteit bezit de α-galactosidase ook transglycosylerings activiteit. Hiermee was het mogelijk om α-galacto-oligosaccharides te produceren, welke eventueel toegepast kunnen worden als prebiotica.

Het screenen van de genomische bank resulteerde ook in de identificatie van twee genen die coderen voor α-glucosidases (AglA en AglB). Beide α-glucosidases behoren tot de familie 13 van de glycosylhydrolases. AglA (EC 3.2.1.10) en AglB (EC 3.2.1.20) werden tot expressie gebracht in *E. coli* en vertoonden allebei hoge hydrolyse activiteit op isomaltose en *p*np-α-glucoside. De K_m

voor *p*np-α-glucoside was respectievelijk 1,05 and 0,47 mM voor AglA en AglB en de V_{max} 228 U en 113 U mg^{-1}. De pH-optimum was voor AglA 6.6 en de temperatuurs-optimum 37 ^{o}C wanneer *p*np-α-glucoside als substraat gebruikt werd. In dit geval waren de waarden voor AglB pH 6,8 en 47 ^{o}C. AglA was zeer actief op isomaltotriose en in mindere mate op threhalose. AglB was actief op maltose en in minder mate op sucrose, een lage activiteit werd waargenomen op melizitose, dextrines met een lage polymerisatie graad, maltitol en maltotriose. De α-glucosidases werden ook getest op transglycosylerings activiteit. AglA synthetiseerde oligosaccharides van trehalose en sucrose. AglB produceerde oligosaccharides van sucrose, maltose en melizitose.

Klonen van de genomische bank van *B. adolescentis* werden opgekweekt in minimaal medium met sucrose als enige koolstofbron (hoofdstuk 4). Een enzymatische fructose dehydrogenase bepaling werd gebruikt om sucrose afbrekende enzymen te identificeren. Plasmiden werden geïsoleerd van kolonies die een positieve reactie gaven. Resultaat van de sequentie analyse was dat twee soorten plasmiden aanwezig waren en dat deze alleen verschillend waren in hun oriëntatie van het gekloneerde fragment in het plasmide. Een open reading frame van 1516 nucleotiden met een hoge mate van homologie voor sucrose fosforylase werd geïdentificeerd op deze fragmenten. Het gen werd *sucP* genoemd en codeert voor een eiwit van 56189 Da. SucP werd heteroloog tot expressie gebracht in *E. coli* en daarna gezuiverd en gekarakteriseerd. Met SDS-PAGE werd de molecuul massa bepaald (58 kDa) evenals met gelpermeatie chromatografie (129 kDa). Hieruit werd geconcludeerd dat het natieve enzym voorkomt als dimeer. Het enzym vertoonde hoge activiteit op sucrose en in mindere mate tegen α-glucose-1-fosfaat. De transglycosylerings eigenschappen van het enzym werden onderzocht door diverse monomeren suikers als acceptor en α-glucose-1-fosfaat als donor te gebruiken. D- en L-Arabinose, D- en L-arabitol evenals xylitol gaven de hoogste productie te zien van transglycosylerings producten. De onderzochte disacchariden en trisacchariden konden niet gebruikt worden als acceptor. De structuur van het transglucosylerings product, verkregen wanneer D-arabinose gebruikt werd als acceptor, werd opgehelderd met NMR. De structuur van dit gesynthetiseerde niet-reducerende dimeer was α-Glc*p*(1→1)β-Ara*f*.

In hoofdstuk 5 wordt de kristalstructuur gegeven van sucrose fosforylase welke tot 1.77 Å resolutie is opgehelderd. Het is het eerste 3D structuur van sucrose fosforylases and is tevens ook de eerste structuur van een fosfaat afhankelijk enzym van de glycosylhydrolase familie 13. De structuur bestaat uit 4 domeinen A, B, B´ en C. Domein A is de typische $(\beta/\alpha)_8$ barrel die voortkomt in familie 13. De katalytische residuen zoals gewoonlijk gevonden in familie 13 (Asp192 en Glu

232) liggen op de uiteinden van de β-sheets 4 en 5 in de $(\beta/\alpha)_8$-barrel. De topologie van het B´-domein zorgt ervoor dat oligosacchariden niet kunnen binden en het reduceert de grootte van het substraat toegangskanaal in vergelijking met andere familie 13 leden, die niet fosfaat afhankelijk zijn. Hiermee wordt onderstreept dat dit domein een modulerende rol speelt in dit enzym. Het is opmerkelijk dat de vouwing van domein C niet teruggevonden wordt in andere onderzochte hydrolases van familie 13. Het gekristalliseerde sucrose fosforylase gedroeg zich als een homodimeer en een contact oppervlakte van 960 Å per monomeer werd berekend voor het dimeer contact. De meeste interacties vonden plaats tussen de twee B-domeinen, maar ook interacties tussen gedeelten van loop 8 van de twee barrels werden waargenomen. Dit resulteert in een grote kloof, die tevens de ingang is naar de twee actieve sites.

Arabinoxylaan arabinofuranohydrolase-D3 (AXHd3) van *B. adolescentis* maakt alleen C3-gekoppelde arabinose eenheden vrij van dubbel gesubstitueerde xylose eenheden. De genomische bank werd onderzocht naar de aanwezigheid van het *axhD3* gen (hoofdstuk 6). Twee plasmiden werden geïdentificeerd die beide een gedeelte van het *axhD3* gen bevatten. De nucleotide sequenties werden gecombineerd en dit resulteerde in drie open reading frames (ORFs). De eerste ORF had hoge homologie met xylanases die behoren tot familie 8 van de glycosylhydrolases en dit gen werd *xylA* genoemd. De tweede open reading frame codeerde voor het *axhD3* gen, wat behoorde tot familie 43 van de glycosylhydrolases. De derde (partiële) ORF codeerde voor een mogelijke carboxylesterase. Alleen het *axhD3* gen werd gekloneerd en tot expressie gebracht in *E. coli*. Verschillende substraten werden gebruikt voor het biochemisch karakteriseren van AXHd3. Het enzym vertoonde de hoogste activiteit op tarwe arabinoxylaan oligosacchariden. Wanneer β-xylanase van *Trichoderma sp*. geïncubeerd werd met oplosbaar tarwe arabinoxylaan dan werd dit substraat verder afgebroken als het eerst voorbehandeld was met AXHd3. De combinatie van AXHd3 en een α-L-arabinofuranosidase van *Aspergillus niger* resulteerde niet in een hoger maximum van vrijgemaakte arabinose eenheden in vergelijking met de incubaties waarin het substraat afzonderlijk met deze enzymen werd geïncubeerd.

Hoofdstuk 7 geeft een overzicht van de koolhydraatmodificerende enzymen van bifidobacteriën. Onlangs werd de genoomsequentie van *B. longum* gepubliceerd en hieruit kon worden afgeleid dat 8% van de geannoteerde genen betrokken waren bij het koolhydraatmetabolisme. Daarnaast werden ook meer sequentie data gepubliceerd van individuele koolhydraatsplitsende enzymen van andere bifidobacteriën-stammen. Dit alles maakte het mogelijk om door middel van deze koolhydraat modificerende enzymen koolhydraten aan te wijzen die mogelijk als prebiotica kunnen dienen. Ook is het mogelijk om aan te geven welke poly- and

oligosacchariden door bifidobacteriën benut zouden kunnen worden. Carbohydrases kunnen ook de potentie hebben om langere of complexere oligosacchariden te maken als deze enzymen transglycosylerings eigenschappen bevatten. De hypothese is dat zulke oligosacchariden gebruikt kunnen worden om de microbiologische compositie te beïnvloeden van de colon. Het voordeel om de eigen enzymen van bifidobacteriën te gebruiken is de waarschijnlijkheid dat de nieuw gesynthetiseerde producten benut kunnen worden door deze micro-organismen. Concepten hoe men koolhydraatmodificerende enzymen kan toepassen in het ontwikkelen en verbeteren van prebiotica worden behandeld.

In hoofdstuk 8 wordt een overzicht gegeven van een aantal glycosylhydrolases van *B. adolescentis* die in de verschillende hoofdstukken (2, 3, 4, 6) in detail zijn bestudeerd en gekarakteriseerd. De hydrolytische activiteit van de glycosylhydrolases van *B. adolescentis* tegen prebiotica zoals arabinoxylaan-oligosacchariden, isomalto-oligosacchariden, arbinogalactaan, and oligosacchariden die sucrose bevatten (raffinose, stachyose, and fructo-oligosacchariden) wordt behandeld. Daarnaast worden er resultaten gegeven van site-directed mutagenese van α-galactosidase om de transglycosylerings activiteit te verbeteren. Tevens wordt bediscussieerd hoe men de enzymatisch gesynthetiseerde oligosacchariden kan toepassen in zuivelproducten.

Tenslotte worden in hoofdstuk 9 de belangrijkste conclusies gegeven van dit proefschrift.

NAWOORD

'Why should you learn when you have everything in life' *Vietnamese farmer 2002*. Deze woorden hebben me de laatste jaren regelmatig bezig gehouden. Waarom zou je inderdaad leren als je het belangrijkste in je leven al hebt? Als ik nu terug kijk op mijn promotieonderzoek dan kan ik toch concluderen dat het de moeite waard is om je te blijven ontplooien en je verder te ontwikkelen. Zoals je kunt zien heb ik de weg van de geleidelijkheid gekozen. Veel mensen hebben in de loop van de jaren aan mij gevraagd: waarom ga je niet promoveren? Fons Voragen was een van die personen. Ik bedank Fons dan ook voor het stimuleren en het bieden van de mogelijkheid om, naast mijn gewone werkzaamheden, dit promotieonderzoek te kunnen uitvoeren bij de Leerstoelgroep Levensmiddelenchemie. Mijn gesprekken met hem over allerlei onderwerpen zoals promotie, de universiteit, de afdeling etc. heb ik altijd als zeer prettig ervaren. Gerrit Beldman was degene die mij veel leerde over koolhydraatsplitsende enzymen. De ruimte die hij me gaf om aan bifidobacteriën te werken heb ik altijd zeer gewaardeerd. Jan Verdoes maakte me wegwijs in de moleculaire technieken en zonder hem was dit proefschrift niet tot stand gekomen.

Verschillende personen hebben gewerkt aan koolhydraatsplitsende enzymen van bifidobacteriën. Ook hen bedank ik graag voor de prettige samenwerking. Katrien Van Laere was de grondlegger van dit werk. Jolanda Ton en Chantal Doeswijk hebben zich beiden verdiept in de transglycosylerings eigenschappen van het α-galactosidase. Sandra Hinz werkte tegelijkertijd aan hetzelfde onderwerp voor haar promotie-onderzoek, waardoor we onze kennis konden delen.

Verder hebben er ook studenten aan het onderwerp gewerkt. Karin Struijs en Annemarie Reijnierse waren de eerste die hebben meegewerkt. Evelien van Boxtel en Raymon Kievit hebben een bijdrage geleverd aan hoofdstuk 4. Door hun doorzettingsvermogen en enthousiasme heeft dit tot publiceerbare gegevens geleid.

Ook heb je specialisten nodig die het mogelijk maken om je verder te verdiepen in de materie. René Verhoef maakte me wegwijs in NMR. A meeting at the 5th Carbohydrate Biogenineerig Meeting in Groningen was the start of a fruitful collaboration with Lars Skov and Desiree Sprogøe, which resulted in the unraveling of the 3D-structure of the sucrose phosphorylase. Barry McCleary from Megazyme and our group shared the same interest for AXHd3, which resulted in chapter 6. I would like to thank them all for their pleasant cooperation.

Daarnaast zijn er ook personen die in de afgelopen periode op een ander vlak hun bijdrage hebben geleverd. Hans Visser gaf, naast zijn gezelligheid op het lab, ook de mogelijkheid om te

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De paranimfen Jean Paul Vincken en Alex van der Heijden hebben ook een grote bijdrage geleverd aan dit proefschrift. Jean Paul en ik werken al bijna even lang bij Wageningen Universiteit en hij is ook degene geweest die mij het laatste zetje gegeven heeft om dit promotieonderzoek te gaan doen. Alex was als vriend altijd op de achtergrond, waarbij we tijdens het sporten konden praten over andere zaken dan wetenschappelijk onderzoek.

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Dia, je wordt als laatste genoemd maar je komt voor mij op de eerste plaats. Jij bent voor mij het allerbelangrijkste.

Ben

CURRICULUM VITAE

Lambertus A. M. van den Broek (Ben) werd op 16 februari 1962 geboren te Ravenstein. In 1978 behaalde hij het MAVO-diploma aan het de PIUS XII MAVO te Ravenstein en in 1980 het HAVO-diploma aan de Vincent van Gogh HAVO te Oss. Vervolgens werd in dezelfde plaats de laboratorium-opleiding HBO-B Biochemie gevolgd aan de HMLS. Na het behalen van zijn diploma in 1983 vervulde hij zijn dienstplicht bij de Koninklijke Marechaussee. In 1985 trad hij in dienst als biochemisch analist bij de afdeling Celfysiologie van de toenmalige Katholieke Universiteit te Nijmegen. In 1988 werd de overstap gemaakt naar de vakgroep Levensmiddelenchemie van de toenmalige Landbouw Universiteit Wageningen. Hij werd in 1997 voor vier maanden gedetacheerd bij de vakgroep Industriële Microbiologie aan dezelfde universiteit. In 1997 werd hij medewerker onderwijs en onderzoek bij de vakgroep Levenmiddelenchemie, waar hij in 2001 startte met het grootste gedeelte van het onderzoek beschreven in dit proefschrift. Het promotieonderzoek aan Wageningen Universiteit werd uitgevoerd naast de normale onderwijs- en beheerstaken voor de vakgroep.

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