CONVERSION OF GDP-MANNOSE INTO VARIOUS GDP-DEOXYHEXOSES IN GRAM-NEGATIVE BACTERIA

Minna Mäki

Rational Drug Design –research program
Biomedicum Helsinki and Haartman Institute
and
Division of General Microbiology
Department of Biosciences
Faculty of Science
University of Helsinki, Finland

Academic dissertation

To be presented, with the permission of the Faculty of Science of the University of Helsinki, for public criticism in the Small Lecture Hall of Haartman Institute (Haartmaninkatu 3, Helsinki) on June 16th, 2003, at 12 o'clock noon.

Helsinki 2003

Supervisor:
Docent Risto Renkonen
Biomedicum Helsinki
Rational Drug Design –research program
and
Haartman Institute
Department of Bacteriology and Immunology
University of Helsinki, Finland

Reviewers:
Docent Marko Salmi
National Public Health Institute Department in Turku
Finland

Professor Mikael Skurnik
Haartman Institute
Department of Bacteriology and Immunology
University of Helsinki, Finland

Opponent:
Professor Jukka Finne
Department of Medical Biochemistry
University of Turku, Finland

ISBN 952-91-5981-1 (paperback)
ISBN 952-10-1242-0 (pdf)
http://ethesis.helsinki.fi
Yliopistopaino
Helsinki 2003

Makeasti oravainen
makaa sammalhuoneessansa;
sinnepä ei Hallin hammas
eikä metsämiehen ansa
ehtineet milloinkaan.

Kammiostaan korkeasta
katselee hän mailman piirii,
taisteloa allans' monta;
havu-oksan rauhan-viiri
päällänsä liepoittaa.

Mikä elo onnellinen
keinuvassa kehtolinnass' !
Siellä kiikkuu oravainen
armaan kuusen äitinrinnass':
Metsolan kantele soi!

Siellä torkkuu heiluhäntä
akkunalla pienoisella,
linnut laulain taivaan alla
saattaa hänen iltasella
unien Kultalaan.

– Aleksis Kivi: Seitsemän veljestä

CONTENTS

ABBREVIATIONS

6dTal	6-deoxy-D-talose
bp	base pair
CF	cystic fibrosis
D-Rha	D-rhamnose
dTDP	deoxythymidine diphosphate
dTTP	deoxythymidine triphosphate
EC	Enzyme Commission
EPS	extracellular polysaccharide
Fuc	L-fucose
FucT	fucosyltransferase
Gal	galactose
GalE	UDP-galactose 4-epimerase
GDP	guanosine diphosphate
GlcNAc	*N*-acetyl-D-glucosamine
GMD	GDP-D-mannose 4,6-dehydratase
GMER	GDP-4-keto-6-deoxy-D-mannose epimerase/reductase
GTS	GDP-6-deoxy-D-talose synthetase
HPLC	high-pressure liquid chromatography
LacNAc	*N*-acetyllactosamine; Galβ1-4GlcNAc
LADII	leukocyte adhesion deficiency type II
Le^a	Lewis A; Galβ1-3(Fucα1-4)GlcNAc
Le^b	Lewis B; (Fucα1-2)Galβ1-3(Fucα1-4)GlcNAc
Le^c	Lewis C; Galβ1-3GlcNAc
Le^x	Lewis X; Galβ1-4(Fucα1-3)GlcNAc
Le^y	Lewis Y; (Fucα1-2)Galβ1-4(Fucα1-3)GlcNAc
LJP	localized juvenile periodontitis
LPS	lipopolysaccharide
MALDI-TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
Neu5Ac	*N*-acetylneuraminic acid
NMR	nuclear magnetic resonance
O-PS	O-polysaccharide
ORF	open reading frame
RMD	GDP-4-keto-6-deoxy-D-mannose reductase
RmlA	glucose-1-phosphate thymidylyltransferase
RmlB	dTDP-D-glucose 4,6-dehydratase
RmlC	dTDP-4-keto-6-deoxy-D-glucose 3,5-epimerase
RmlD	dTDP-4-keto-6-deoxy-L-mannose reductase
SDR	short chain dehydrogenase/reductase
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel eletrophoresis

Sialyl Le^a	Sialyl Lewis A; Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAc
Sialyl Le^x	Sialyl Lewis X; Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc
S-layer	surface-layer
SPA	specific polysaccharide antigen
SPE	solid-phase extraction
spp	species
TEAA	triethylammoniumacetate
Tll	dTDP-4-keto-6-deoxy-L-mannose reductase
UDP	uridine diphosphate

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by their Roman numerals. In addition, some unpublished data are presented.

I **Järvinen, N., Mäki, M., Räbinä J., Roos, C., Mattila, P., and Renkonen, R.** (2001) Cloning and expression of *Helicobacter pylori* GDP-L-fucose synthesizing enzymes (GMD and GMER) in *Saccharomyces cerevisiae*. *European Journal of Biochemistry.* **268**,6458-64.

II **Mäki, M., Järvinen, N., Räbinä, J., Roos, C., Maaheimo, H., Mattila, P., and Renkonen, R.** (2002) Functional expression of *Pseudomonas aeruginosa* GDP-4-keto-6-deoxy-D-mannose reductase (RMD) which synthesizes GDP-D-rhamnose. *European Journal of Biochemistry.* **269**, 593-601.

III **Räbinä, J., Mäki, M., Savilahti, E.M., Järvinen, N., Penttilä, L., and Renkonen, R.** (2001) Analysis of nucleotide sugars from cell lysates by ion-pair solid-phase extraction and reversed-phase high-performance liquid chromatography. *Glycoconjugate Journal.* **18**, 799-805.

IV **Mäki, M., Järvinen, N., Räbinä, J., Maaheimo, H., Mattila, P., and Renkonen, R.** (2003) Cloning and functional expression of a novel GDP-6-deoxy-D-talose synthetase from *Actinobacillus actinomycetemcomitans*. *Glycobiology*. **13**, 295-303.

SUMMARY

Multiple drug-resistance of pathogens creates a high demand for drugs, which act with new strategies. Inhibition of host – pathogen interaction is a potential target for interventions of novel antibacterial therapeutics. Cell surface polysaccharides situated at the interface between the bacterium and its environment have important roles in environment – bacteria interaction, especially in host – pathogen interactions. Hence, manipulation of the biosynthesis of bacterial cell surface polysaccharides may influence the interplay between host and pathogen.

The biosynthesis of cell surface glycoconjugates involves specific glycosyltransferases utilizing nucleotide sugars as activated donors. Recently, an interest in the nucleotide sugar metabolism of bacteria has been increased, because bacterial cell surface polysaccharides contain unique monosaccharides. Hence, the biosynthetic pathways of these monosaccharides could be considered as potential targets for antibacterial agents. Drugs inhibiting the enzymes involved in the nucleotide sugar pathways specific for bacteria might not interfere with human metabolic pathways and thus might not be harmful for humans. However, detailed studies are needed to fully understand the enzymology related to bacterial nucleotide sugar metabolism.

In this study, the nucleotide sugar synthesizing enzymes, that convert GDP-D-mannose into GDP-L-fucose, GDP-D-rhamnose and GDP-6-deoxy-D-talose, were identified and characterized at molecular level. GDP-D-mannose 4,6-dehydratase (GMD) and GDP-4-keto-6-deoxy-D-mannose 3,5-epimerase/reductase (GMER) enzymes synthesizing GDP-L-fucose were characterized from *Helicobacter pylori*, GDP-D-rhamnose synthesizing GDP-4-keto-6-deoxy-D-mannose reductase (RMD) was characterized from *Pseudomonas aeruginosa*, and GDP-6-deoxy-D-talose synthesizing GDP-6-deoxy-D-talose synthetase (GTS) was characterized from *Actinobacillus actinomycetemcomitans* serotype a. These microorganisms are Gram-negative pathogens, in which deoxyhexoses L-fucose, D-rhamnose and 6-deoxy-D-talose are constituents of LPS. In Gram-negative bacteria, LPS molecules are major integral components of the outer membrane, which faces the environment surrounding the bacteria.

In addition, a solid-phase extraction and an ion-pair reversed-phase high-pressure liquid chromatography methods were developed for sample preparation and analysis of synthesized nucleotide sugars, respectively.

1. INTRODUCTION

Cell surface polysaccharides are situated at the interface between a bacterium and its environment thus having important roles in environment – bacteria interplay, especially in host – pathogen interplay. In Gram-negative bacteria, lipopolysaccharides (LPS) are major integral components of the outer membrane facing the environment surrounding the bacteria. LPS has been shown to play a role in the variety of biological effects associated with Gram-negative sepsis. Furthermore, LPS contribute to the structural properties of the cell envelope and provide a selective permeability barrier. LPS consist of three different components: lipid A, core oligosaccharide and O-antigen (reviewed in Lerouge and Vanderleyden, 2002; Raetz and Whitfield, 2002).

O-antigen, which is the most exposed part of the LPS, consists of repeating units made up of varying, usually from three to five, numbers of monosaccharides. The structural diversity of O-antigens is remarkable; The O-antigen can be a linear or branched homopolymer or, more frequently, a heteropolymer. The O-antigen can also be modified nonstoichiometrically with glycosylation or by the addition of noncarbohydrate substituents, *e.g.* by acetylation and methylation. The chemical structure of O-antigen can be strain-specific, but it can also vary within a single strain. The primary role of O-antigen appears to be protective. The exact role of O-antigen in different bacteria is variable and can be potentially magnified in bacteria that can produce more than one chemically distinct O-antigen (reviewed in Lerouge and Vanderleyden, 2002; Raetz and Whitfield, 2002).

For decades tremendous efforts have been made to determine the exact chemical structure of various O-antigen molecules. Recently, much effort has also been put towards elucidating the enzymology involved in O-antigen biosynthesis. O-antigens are synthesized from nucleotide sugars by specific glycosyltransferases that catalyze the transfer of monosaccharides to the growing oligo- or polysaccharide structure (Figure 1). In general, there is at least one specific glycosyltransferase for each monosaccharide and specific linkage formed between a monosaccharide and an acceptor molecule. To date, a large number of prokaryotic glycosyltransferases with different donor, acceptor and linkage specificities have been cloned and characterized (Unligil and Rini, 2000).

Nucleotide sugars are activated sugar metabolites which act as donors in glycosylation reactions. Recently, interest in the nucleotide sugar metabolism of bacteria has increased, because bacterial cell surface polysaccharides contain unique monosaccharides that are not found in humans and because enzymes involved in the conversions of nucleotide sugars are potential targets for manipulating substrate levels available for polysaccharide glycosyltransferases. Manipulation of the biosynthesis of bacterial cell surface polysaccharides may influence the host – pathogen interaction or even the viability of a bacterium. Hence, the biosynthetic pathways of bacterial nucleotide sugars could be considered as potential targets for interventions of antibacterial therapeutics. These enzymes do not have human homologs thus drugs,

which inhibit them might not interfere with metabolic pathways in humans. However, more detailed studies are needed to fully understand the enzymology related to bacterial nucleotide sugar metabolism.

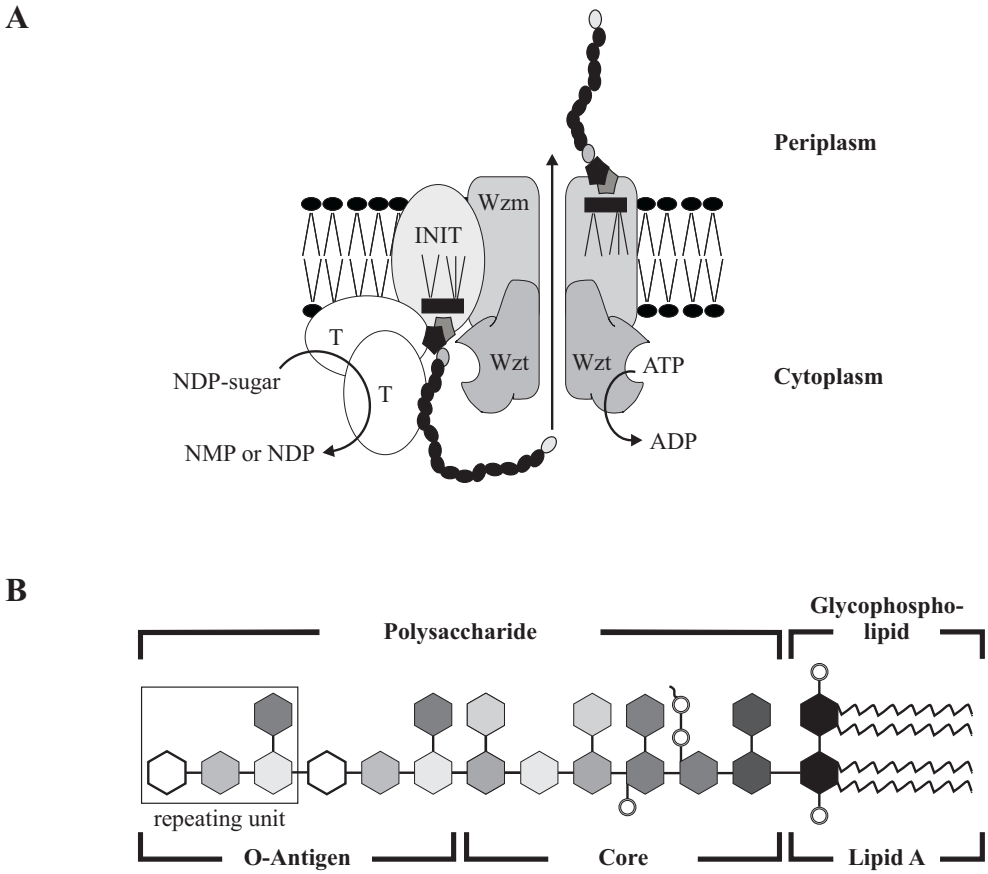

Figure 1. Panel A illustrates a model for linear O-antigen assembly via the ABC-transporter dependent mechanism. The assembly requires NDP-sugar (nucleotide diphosphate sugar), specific glycosyltransferase enzymes (T) and an initiating enzyme (INIT). The ABC-transporter formed by the Wzm and Wzt proteins is required for transfer of the O-antigen polymer to the periplasmic face of the inner membrane, where the LPS assembly is completed before translocation to the outer membrane of a bacterium (reviewed in Lerouge and Vanderleyden, 2002; Raetz and Whitfield, 2002). NMP, nucleoside monophosphate; NDP, nucleoside diphosphate. **Panel B illustrates the schematic presentation of the architecture of branched, heteropolymeric LPS.** Noncarbohydrate substituents may be present in nonstoichiometric amounts, *e.g.* fatty acids (zig-zag lines) and phosphoryl residues (circles) (reviewed in Lerouge and Vanderleyden, 2002; Raetz and Whitfield, 2002).

In this thesis, the cloning and functional characterization of enzymes involved in the synthesis of nucleotide sugars, GDP-L-fucose, GDP-D-rhamnose and GDP-6-deoxy-D-talose, are described. Enzymes were cloned from the Gram-negative pathogens *Helicobacter pylori*, *Pseudomonas aeruginosa* and *Actinobacillus actinomycetemcomitans*, which contain L-fucose, D-rhamnose and 6-deoxy-D-talose, respectively, as constituents of their LPS O-antigen or distinct O-polysaccharide (also known as capsular polysaccharide).

L-fucose, D-rhamnose and 6-deoxy-D-talose are 6-deoxyhexoses. In general, deoxysugars are an important class of carbohydrates that are formed from common monosaccharides by replacement of one or more hydroxyl groups with hydrogen, *e.g.* in the formation of 6-deoxyhexoses the C6 carbon is deoxygenated. 6-deoxyhexoses are formed from nucleoside diphosphate-activated hexoses via a 4-keto-6-deoxy intermediate (Figure 2). In addition to LPS, deoxysugars can also be found in extracellular polysaccharides (EPS) and antibiotics in bacteria (reviewed in Johnson and Liu, 1998; Trefzer *et al*., 1999).

2. L-FUCOSE PATHWAY

2.1. Prevalence of fucose

Fucose is a deoxyhexose sugar found widely in the glycoconjugates of prokaryotes and eukaryotes. Of the two isomers, L and D, the former is much more common and the latter is rare and not found in mammals. D-fucose is found in a limited number of both Gram-negative and Gram-positive bacteria, in which it is a constituent of the cell wall and capsule structures. The activated nucleotide sugar form of D-fucose is dTDP-D-fucose (Figure 2), which is synthesized from dTDP-D-glucose via dTDP-4-keto-6-deoxy-D-glucose by dTDP-D-glucose 4,6-dehydratase (RmlB, EC 4.2.1.46) and dTDP-4-keto-6-deoxy-D-glucose reductase (EC not yet classified) (Yoshida *et al*., 1999). The activated nucleotide sugar form of L-fucose is GDP-L-fucose, of which the biosynthetic pathway (Figure 3) is described in detail in section 2.3.

L-fucose is an important component of the complex glycoconjugates of species ranging from bacteria to mammals. In humans, L-fucose is an essential component of *N*-linked and *O*-linked glycoproteins and glycolipids that mediate intercellular adhesion and recognition processes and thereby play a role in immunity, inflammation and metastasis of malignant cells (reviewed in Lowe, 2001; Tonetti *et al*., 1998). Specifically, L-fucose is present in the human ABO blood group antigens and Lewis glycans. For example, the sialyl Lewis X glycans, a crucial decoration of selectin ligands have been demonstrated to have a role in leukocyte rolling and extravasation into tissues (reviewed in Lowe, 1997).

L-fucose is also a constituent of the cell wall and capsule structures of Gram-negative and Gram-positive bacteria. For instance, most *E. coli* strains as well as the other species of the *Enterobacteriaceae* family are known to produce L-fucose containing EPS colanic acid (reviewed in Whitfield and Roberts, 1999). In addition, L-fucose has

Table 1. Prevalence of L- and D-isomers of fucose, rhamnose and 6-deoxy-talose in humans and in various strains of microorganisms used in this study

	D-mannose	**D-fucose**	**L-fucose**	**D-rhamnose**	**L-rhamnose**	**6-deoxy-D-talose**	**6-deoxy-L-talose**
H. pylori	+ [a]	-	+ [a]	+ [b]	-	-	-
P. aeruginosa	+ [c]	-	-	+ [c]	+ [c]	-	-
A. actinomycetemcomitans	+ [d]	+ [e]	-	-	+ [e]	+ [d]	+ [d]
E. coli	+ [f]	-	+ [f]	-	+	-	+
S. cerevisiae	+ [g]	-	-	-	-	-	-
Human	+ [f]	-	+ [f]	-	-	-	-

References: ([d]Amano *et al*., 1989; [g]Hashimoto *et al*., 1997; [b]Kocharova *et al*., 2000; [e]Perry *et al*., 1996; reviewed in [c]Rocchetta *et al*., 1999; [f]Tonetti *et al*., 1998; [a]Wang *et al*., 2000)

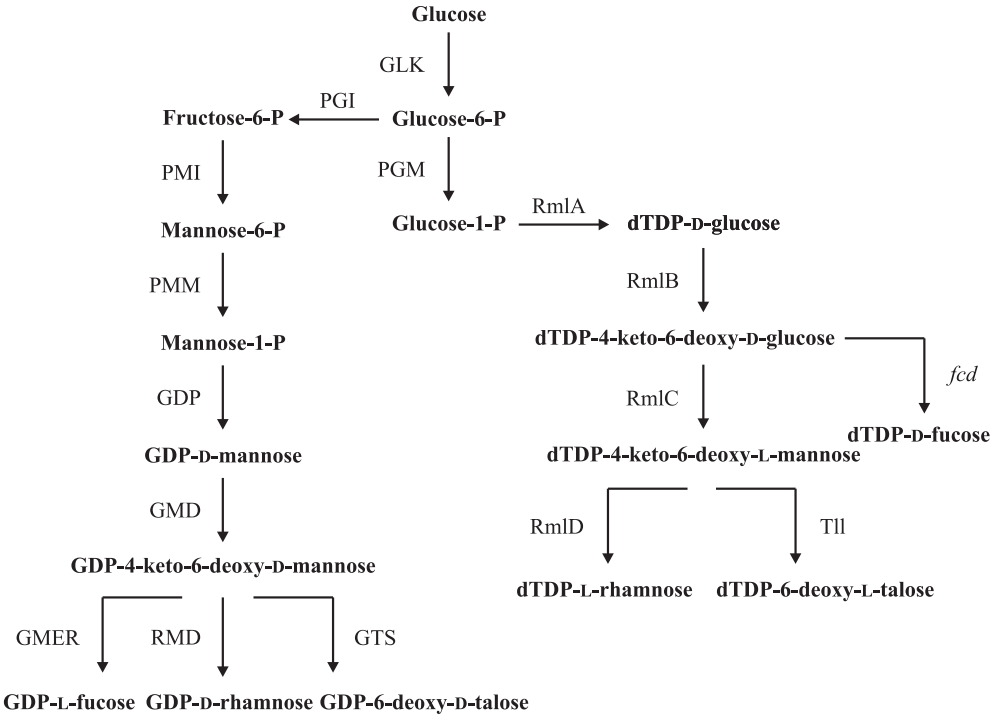

Figure 2. Proposed biosynthetic pathways for nucleotide sugar precursors for L- and D-isomers of fucose, rhamnose and 6-deoxy-talose. GLK, glucokinase (EC 2.7.1.2); PGI, phosphoisomerase (EC 5.3.1.9); PMI, Phosphomannoisomerase (EC 5.3.1.8); PMM, phosphomannomutase (EC 5.4.2.8); GDP, manno-1-phosphate guanylyltransferase (EC 2.7.7.22); GMD, GDP-D-mannose 4,6-dehydratase (EC 4.2.1.47); GMER, GDP-4-keto-6-deoxy-D-mannose 3,5-epimerase/reductase (EC not yet classified); RMD, GDP-4-keto-6-deoxy-D-mannose reductase (EC 1.1.1.187); GTS, GDP-6-deoxy-D-talose synthetase (EC 1.1.1.187); PGM, phosphoglycomutase (EC 5.4.2.2); RmlA, glucose-1-phosphate thymidylyltransferase (EC 2.7.7.24); RmlB, dTDP-D-glucose 4,6-dehydratase (EC 4.2.1.46); RmlC, dTDP-4-keto-6-deoxy-D-glucose 3,5-epimerase (EC 5.1.3.13); RmlD, dTDP-4-keto-6-deoxy-L-mannose reductase (EC 1.1.1.133); Tll, dTDP-4-keto-6-deoxy-L-mannose reductase (EC 1.1.1.133); gene product of *fcd,* dTDP-4-keto-6-deoxy-D-glucose reductase (EC not yet classified); GDP, guanosine diphosphate; dTDP, deoxythymidine diphosphate. Because the nomeclature of these enzymes is not uniform, the Enzyme Commission (EC) numbers are also represented. In the text, enzymes are referred to by the names mentioned in this figure. However, it is worth mentioning that according to the bacterial polysaccharide gene nomenclature (BPGN) the recommended name for the enzyme responsible for GDP-4-keto-6-deoxy-D-mannose 3,5-epimerase/reductase (GMER) activity is Fcl (Reeves *et al*., 1996). In this thesis, GMER is used to refer to GDP-4-keto-6-deoxy-D-mannose 3,5-epimerase/reductase both in prokaryotes and in eukaryotes.

been shown to be a substantial cell wall component of several human pathogens, *e.g. H. pylori* (reviewed in Appelmelk *et al*., 2000), *E. coli* O:157 (Barua *et al*., 2002; Wang and Reeves, 1998), *Campylobacter fetus* (Moran *et al*., 1994), *Yersinia enterocolitica* O:8 and several serotypes of *Y. pseudotuberculosis* (reviewed in Skurnik and Zhang, 1996). L-fucose is also a structural component of the Nod-factors that are important signals in the nodulation of the genera of plant microbes *Azorhizobium* and *Rhizobium* (reviewed in Carlson *et al*., 1994; Trefzer *et al*., 1999).

2.2. L-fucose in *H. pylori* LPS

H. pylori, a microaerophilic bacterium, is an important human gastric pathogen, infecting about half of the world's population. *H. pylori* is a major cause of chronic gastritis and plays a role in the other gastric diseases like duodenal ulcers, gastric ulcers, gastric cancer and gastric lymphoma (reviewed in Marshall, 2002). The O-antigen of LPS in most *H. pylori* strains contains fucosylated Lewis glycans, predominantly the type II antigens Lewis X (Le^x) and Lewis Y (Le^y) (Figure 6). Serologically, 80-90% of *H. pylori* strains express Le^x and/or Le^y epitopes (Simoons-Smit *et al*., 1996; Wirth *et al*., 1996). In addition, i-antigen, blood group A antigen, sialyl Le^x, Lewis disaccharide, and the type I antigen like Le^a, Le^b, and H Type I antigen (Figure 6) have been found in the O-antigen of *H. pylori* LPS (Table 2). Lewis antigens expressed by *H. pylori* are structurally similar to human Lewis blood group antigens. This restricted diversity in the O-antigen expression of *H. pylori* is unusual and suggests a role for specific epitopes in pathogenesis. Additionally, *H. pylori* Lewis antigens can undergo phase variation, which is the random, reversible high-frequency switching of phenotype within a single strain (reviewed in Wang *et al*., 2000). A recent study demonstrated that acidic environment can trigger phase variation from Le^x to Le^y expression (Moran *et al*., 2002). The antigenic phase variation is most probably beneficial to *H. pylori* in adaptation to different environments and environmental changes, but its role in colonization and pathogenesis is unclear. However, phase variation has been demonstrated to contribute to the virulence of bacteria like *Neisseria* spp. (van Putten, 1993) and *Haemophilus influenza*e (Humphries and High, 2002; Weiser and Pan, 1998).

Table2. The chemically characterized Lewis structures of *H. pylori* strains

***H. pylori* strain**	**Lewis glycan**	**Reference**
26695	Le^x, Le^y, Le disaccharide, Linear Blood group	(Monteiro *et al*., 2000)
J99	Le^x, Le^y	(Monteiro *et al*., 2000)
P466	Le^x, sialyl Le^x, Le^y	(Monteiro *et al*., 2000)
NCTC11637	Le^x, Le^y, H Type I	(Aspinall *et al*., 1996)
NCTC11639	Le^x	(Wang *et al*., 2000)
UA802	Le^y	(Wang *et al*., 1999)
UA948	Le^a, Le^y	(Monteiro *et al*., 1998)
UA915	Le^b, H Type I	(Monteiro *et al*., 2000)
UA1111	Le^a, Le^b, H Type I	(Wang *et al*., 2000)

H. pylori – host interaction is under extensive study, and the whole genome sequences of two *H. pylori* strains have been determined (Alm *et al*., 1999; Tomb *et al*., 1997). During the studies several hypotheses have been made for the possible roles of Lewis antigens in the course of colonization and/or persistent infection of *H. pylori*. The molecular mimicry of human Lewis antigens has been proposed to protect *H. pylori* from antigen-specific host defences and thus aid the persistence of its infection. Furthermore, the *H. pylori* Lewis antigen have been suggested to induce autoantibodies against the host gastric epithelia and cause autoimmune-mediated inflammation and thereby contribute to the *H. pylori* infection. However, there are contradictory observations about these hypotheses. Currently, the best evidence for the role of *H. pylori* Lewis glycans is that they may play a role in the adhesion or colonization of the bacterium to the host gastric mucosa (reviewed in Appelmelk *et al*., 2000; Gerhard *et al*., 2002; Moran and Prendergast, 2001). Especially, *H. pylori* Le^x has been suggested to mediate adhesion to gastric epithelial cells and to be essential for colonization (Appelmelk and Vandenbroucke-Grauls, 2000; Edwards *et al*., 2000; Martin *et al*., 2000; Osaki *et al*., 1998).

2.3. GDP-L-fucose metabolism

GDP-L-fucose is synthesized *in vivo* via two different metabolic pathways. In the salvage pathway, which is not found in bacteria, GDP-L-fucose is synthesized from L-fucose derived from exogenous sources by the action of a L-fucose kinase (EC 2.7.1.52) and a GDP-fucose pyrophosphorylase (EC 2.7.7.31) (reviewed in Bulter and Elling, 1999).

The *de novo* pathway of GDP-L-fucose (Figure 3), which is evolutionarily conserved, is more prominent. It was first identified in bacteria in the 1960s (Ginsburg, 1960), and then described in mammals (Overton and Serif, 1981), invertebrates (Bulet *et al*., 1984) and plants (Liao and Barber, 1972). This pathway starts from GDP-D-mannose, which is converted into GDP-L-fucose in three steps by two enzymes. The first reaction step is a dehydration of GDP-D-mannose, which leads to the formation of an intermediate product, GDP-4-keto-6-deoxy-D-mannose, and which is catalyzed by a GDP-D-mannose 4,6-dehydratase (GMD, EC 4.2.1.47) (reviewed in Allard *et al*., 2002). GMD catalyzes oxidation of the C4 group of the mannose ring to a keto form and the subsequent reduction of the C6 group to a methyl group. During the concerted action of oxidation and reduction an intramolecular hydride transfer occurs from the C4 position to the C6 position on the mannose ring by a tightly bound NADP cofactor, which is essential for the activity of GMD (Oths *et al*., 1990).

In the second reaction step the intermediate product, GDP-4-keto-6-deoxy-D-mannose, subsequently undergoes epimerisation at positions C3 and C5, which leads to a change of D- to L-configuration and the formation of GDP-4-keto-6-deoxy-L-galactose. Finally, a NADPH-dependent reduction of the keto group at the C4 position of GDP-4-keto-6-deoxy-L-galactose leads to the formation of GDP-L-fucose. The last two steps of the pathway are catalyzed by a bifunctional enzyme GDP-4-keto-6-deoxy-D-mannose 3,5-epimerase/reductase (GMER, also known as Fcl/WcaG in *E. coli* and FX in man, EC not yet classified) (reviewed in Allard *et al*., 2001). GMER can utilize NADH or

NADPH as a cofactor, but NADPH has been demonstrated to be more substantial than NADH (Menon *et al*., 1999).

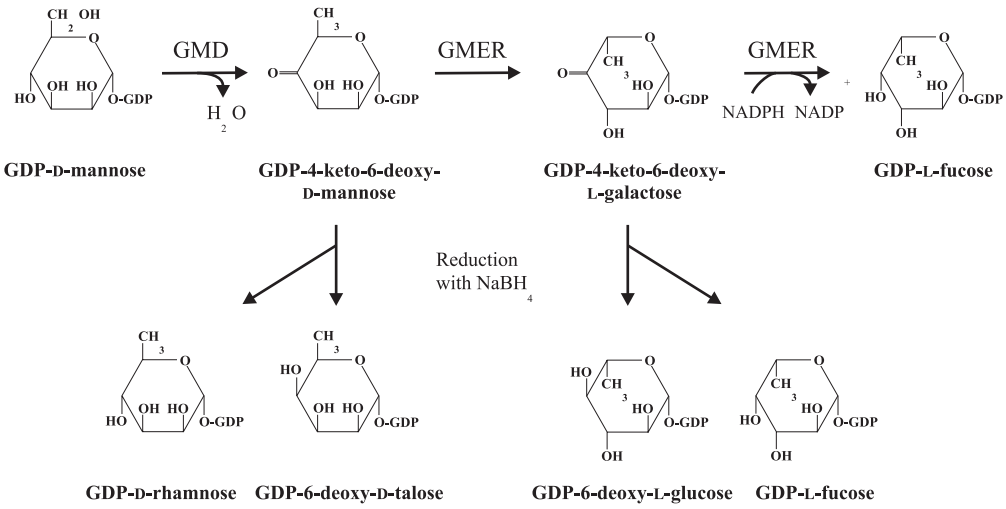

Figure 3. Schematic presentation of the *de novo* pathway of GDP-L-fucose and the products obtained after chemical reduction of intermediate products of the pathway. The intermediate products of the *de novo* pathway of GDP-L-fucose can be studied by reducing them chemically with sodium borohydride ($NaBH_4$). The chemical reduction of GDP-4-keto-6-deoxy-D-mannose leads to the formation of GDP-D-rhamnose and GDP-6-deoxy-D-talose and reduction of GDP-4-keto-6-deoxy-L-galactose leads to the formation of GDP-6-deoxy-L-glucose and GDP-L-fucose. GMD, GDP-D-mannose 4,6-dehydratase; GMER, GDP-4-keto-6-deoxy-D-mannose 3,5-epimerase/reductase; GDP, guanosine diphosphate.

2.4. GDP-D-mannose 4,6-dehydratase (GMD)

GMDs have been obtained at various degrees of purity from different sources, both from bacteria like *Klebsiella pneumonie* (Yamamoto *et al*., 1993) and mammals like porcine thyroid (Broschat *et al*., 1985). Cloning of the *gmd* gene and characterization of the corresponding enzyme have been performed from human (Ohyama *et al*., 1998; Sturla *et al*., 1997; Sullivan *et al*., 1998), *Arabidopsis thaliana* (known as MUR1*)* (Bonin *et al*., 1997) and bacteria like *E. coli* (Sturla *et al*., 1997). Multimerisation of the purified and cloned GMDs vary as well as the molecular masses of the monomers (*ca*. 40-55 kDa). In general, the reported mature GMDs are homodimers consisting of two *ca.* 42 kDa subunits. An amino acid sequence GILFNHES is commonly found in the characterized GMDs, but the possible function of this sequence is unknown.

Recently, the X-ray crystallographic structure of *E. coli* GMD has been determined at 2.3 Å resolution (Somoza *et al*., 2000). The resolved GMD structure was shown to be a homodimeric protein with two *ca*. 42 kDa monomers composed of two domains (Figure 4). The larger N-terminal domain contains the NADP(H) cofactor binding site

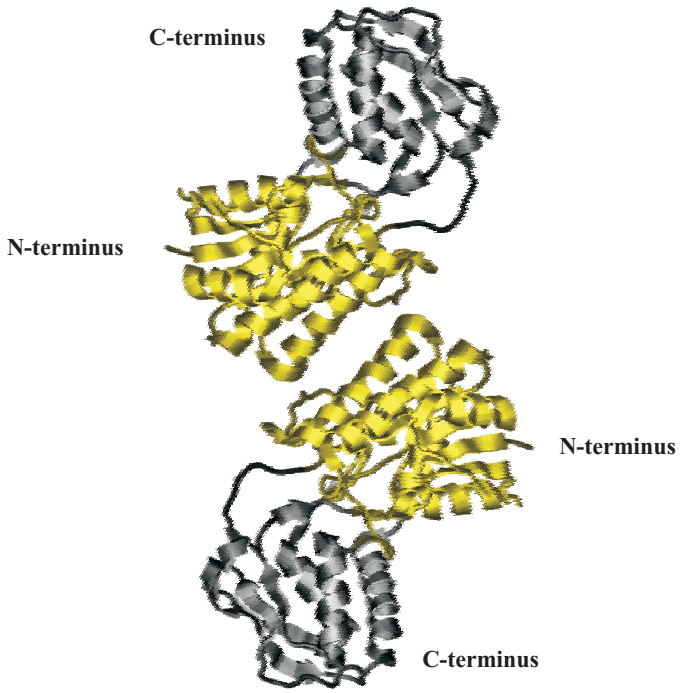

Figure 4. Illustration of the three-dimensional structure of *E. coli* GMD. *E. coli* GMD is a homodimeric protein, of which monomers are composed of two domains. The N-terminal, classical Rossman fold (seven parallel β-sheets and seven α-helices), that binds the cofactor, is illustrated in yellow, whereas the C-terminal, substrate binding domain is dark grey. The N- and C-termini of the enzyme are indicated.

in a classical Rossman fold topology consisting of seven parallel β-sheets and seven α-helices. The smaller C-terminal domain harbors the binding site for the nucleotide sugar substrate. This domain is predominantly composed of four β-sheets and a cluster of three parallel α-helices. These two domains form a pocket where the cofactor and the substrate bind, and catalysis of the reaction occurs. Both subunits are suggested to be catalytically competent. Somoza and colleagues (2000) have also hypothesized that the quaternary structures of GMD family is likely to be conserved, with the exception of the some C-terminal areas that are not involved in nucleotide sugar binding or in catalysis. The hypothesis is based on the sequence identities, which range from 40-99% among the GMD family. The dimeric structures have already been reported for the *K. pneumonie* (Yamamoto *et al*., 1993) and human GMDs (Bisso *et al*., 1999). However, the recently resolved three-dimensional structure of *A. thaliana* GMD (named as MUR1) is a tetramer (Mulichak *et al*., 2002) and there are reports suggesting that *H. pylori* GMD is also a tetramer (Wu *et al*., 2001) and that porcine thyroid GMD

might be a hexamer (Broschat *et al*., 1985) being in contradiction to the hypothesis proposed by Somoza and colleagues (2000).

The *E. coli* GMD model shows that GMD is a member of a short chain dehydrogenase/ reductase (SDR) protein family. The SDR protein family is a very large family of enzymes, most of which are known to be NADH/NADPH-dependent oxidoreductases (Jörnvall *et al*., 1995). The three-dimensional structure of *E. coli* GMD is closely related to the resolved homodimeric structures of *E. coli* GMER (Rosano *et al*., 2000) and *E. coli* UDP-galactose 4-epimerase (GalE) (Thoden *et al*., 1996), both of which are also members of the SDR protein family. GalE catalyzes the interconversion of UDP-galactose and UDP-glucose. In addition, the GMD model and site-directed mutagenesis studies show that the Ser-Tyr-Lys catalytic triad common to enzymes of the SDR protein family is found in *E. coli* GMD, with the exception that Thr replaces Ser. Most of GMDs have also Thr in the usual place of Ser in the catalytic triad. This replacement of Ser to Thr is an unique feature among the SDR protein family. However, the influence of the exchange of Ser to Thr in GMDs is not known. Somoza and coworkers (2000) also observed that the dimerisation of GMD happens in a similar way as has been described for the other members of SDR protein family. On the basis of the GMD model and mutational analysis, the Glu135 is proposed to play a role in the deprotonation/reprotonation of the mannose ring thus acting as an active-site base during the catalysis (Somoza *et al*., 2000).

GDP-L-β-fucose, which is the end product of the pathway, has been demonstrated to inhibit the activity of both human and bacterial GMD via a specific feedback inhibitory mechanism (Bisso *et al*., 1999; Sturla *et al*., 1997). In *E. coli* GMD, GDP-L-β-fucose also shows competitive inhibition in respect to GDP-D-mannose indicating that GDP-L-β-fucose probably binds to the same site on the enzyme as GDP-D-mannose. The competitive inhibition also indicates that GDP-L-β-fucose regulates its own biosynthesis through the activity of GMD (Somoza *et al*., 2000). In contrast, GDP-L-β-fucose shows no competitive inhibition in human GMD (Bisso *et al*., 1999). GDP-L-α-fucose, an anomer of GDP-L-β-fucose, does not inhibit the activity of human and bacterial GMD, whereas GDP-D-glucose shows an inhibitory effect (Bisso *et al*., 1999; Sturla *et al*., 1997).

A defective synthesis of GDP-L-fucose and especially defects in GMD activity has been linked to stem shoot development in plants (Bonin *et al*., 1997). Humans deficient in fucosylation suffer from the rare immune disorder leukocytes adhesion deficiency type II (LAD II) that is characterized by defective selectin ligand formation, recurrent infections and severe mental and growth retardation (Becker and Lowe, 1999). Recently, the molecular mechanism for this disorder has been located to the GDP-L-fucose transporter gene (Etzioni *et al*., 2002; Luhn *et al*., 2001). In contrast, increased fucosylation of glycoconjugates, *e.g.* increased expression of sialyl Le^x or sialyl Le^a has been observed in metastasis of cancer patients (Martin-Satue *et al*., 1998; Ura *et al*., 1997) or inflammatory diseases, such as acute transplant rejection or bronchial asthma (Kirveskari *et al*., 2000; Toppila *et al*., 2000; Toppila *et al*., 1999).

2.5. GDP-4-keto-6-deoxy-D-mannose 3,5-epimerase/reductase (GMER)

GMER was first purified from porcine thyroid (Chang *et al*., 1988) followed by cloning the corresponding gene from humans (named as FX) (Tonetti *et al*., 1996), *E. coli* (also named as Fcl/WcaG) (Tonetti *et al*., 1998) and *A. thaliana* (named as GER1) (Bonin and Reiter, 2000). Mature human and *E. coli* GMERs are homodimers of two *ca*. 34 kDa subunits. The monomer of *A. thaliana* GMER is also *ca*. 35 kDa, but its possible multimerisation is unknown.

Recently, two research groups have determined by the X-ray crystallography the structure of *E. coli* GMER at 2.1-2.2 Å resolution (Rizzi *et al*., 1998; Somers *et al*., 1998). Furthermore, the comparison of the crystal structures of the native and three mutated GMERs have been elucidated at 1.45-1.60 Å resolution (Rosano *et al*., 2000). The resolved GMER structure was shown to be a homodimeric protein with each monomer composed of two domains (Figure 5). The N-terminal domain contains NADP(H) cofactor binding site in a modified Rossman fold topology containing six parallel β-sheets and six α-helices. The C-terminal domain harbors the binding site for the nucleotide sugar substrate. This domain is composed of three parallel α-helices and two antiparallel pairs of parallel β-sheets. This GMER model shows that GMER is a member of the SDR protein family and it is closely related to the resolved

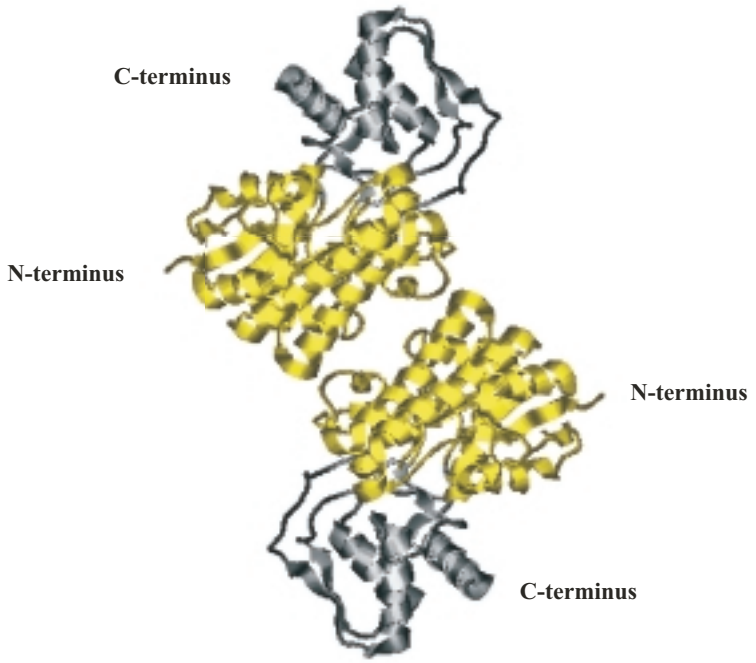

Figure 5. Illustration of the three-dimensional structure of *E. coli* GMER. *E. coli* GMER is a homodimeric protein, of which monomers are composed of two domains. The N-terminal, modified Rossman fold (six parallel β-sheets and six α-helices), that binds the cofactor, is illustrated in yellow, whereas the C-terminal, substrate binding domain is dark grey. The N- and C-termini of the enzyme are indicated.

homodimeric structures of *E. coli* GMD (Somoza *et al*., 2000) and *E. coli* GalE (Thoden *et al*., 1996). Additionally, the GMER model and site-directed mutagenesis studies show that the catalytic mechanism of GMER is based on the concerted action of Ser-Tyr-Lys residues forming the conserved catalytic triad of the SDR protein family. The GMER model also indicates that the GDP-4-keto-6-deoxy-D-mannose substrate might be located in the pocket facing Ser107, Ser108, Cys109 and His179 residues thus bringing the C4 position of the 4-ketopyranose rings close to the nicotinamide moiety of NADPH during reduction (Rizzi *et al*., 1998; Rosano *et al*., 2000). Moreover, the Cys109 and His179 residues are suggested to play a primary role in the epimerisation reaction (Rosano *et al*., 2000).

Among the SDR protein family, the ability of GMER to catalyze two different reactions, epimerisation and reduction, at the same active site appears unique. Furthermore, it is not common in the NADH/NADPH-dependent SDR protein family that the epimerisation catalyzed by GMER occurs in the absence of its cofactor NADPH (Menon *et al*., 1999). The bifunctional nature of GMER also separates the GDP-L-fucose biosynthesis from that of other deoxy or dideoxy sugars, in which the epimerisation and reduction reactions are catalyzed by separate enzymes.

Defects in GMER activity has been linked to nodulation (Lamrabet *et al*., 1999; Mergaert *et al*., 1997) and survival of bacteria (McGowan *et al*., 1998). In *H. pylori*, the *gmer* gene (also known as *wbcJ*) is upregulated in response to an acidic environment. The acid-induced upregulation suggests that an acidic pH may stimulate LPS synthesis, which may be substantial in decreasing the host gastric acid secretion and in masking the antigenic surface epitopes of *H. pylori* and thereby facilitating early colonization (McGowan *et al*., 1998). Moreover, the *H. pylori gmer* knock-out mutant is demonstrated to be more sensitive to acid stress than the wild-type strain suggesting that O-antigen expression might contribute to acid survival of *H. pylori* (McGowan *et al*., 1998).

2.6. Fucosyltransferases (FucT)

GDP-L-fucose and dTDP-D-fucose are used as substrates for specific fucosyltransferases that are responsible for transferring L-/D-fucose at different positions of an acceptor molecule. To date, no D-fucosyltransferases has been identified, whereas ten human L-fucosyltransferases (FucT) responsible for α1,2-, α1,3-, α1,4-, or α1,6-linkages on glycans (FutTs I-IX) (reviewed in de Vries *et al*., 2001) or *O*-linkage to Ser or Thr have been identified (O-FucT-1) (Wang *et al*., 2001). In addition, Roos and colleagues (2002) have proposed candidates representing two novel human FucTs named as FutX and FutXI. The candidates are proposed on the basis of *in silico* analysis and they have not been experimentally verified. FucTs responsible for α1,2-, α1,3-, α1,4-, or α1,6-linkages on glycans have been identified only in a limited number of bacteria, *e.g. H. pylori* and some *Rhizobium* species (reviewed in Oriol *et al*., 1999; Wang *et al*., 2000).

Three *futT* genes, *i.e.* two α1,3-*fucTs* (or α1,3/4-*fucTs*) and one α1,2-*fucT* are present in each *H. pylori* isolate investigated to date (Alm *et al*., 1999; Rasko *et al*., 2000;

Tomb *et al*., 1997; Wang *et al*., 1999). The encoded FucTs from different *H. pylori* strains have varying levels of activity and different acceptor specificities.

As in mammalian cells, the synthesis of Lewis antigens in *H. pylori* is directed by series of glycosyltransferases that act sequentially upon a precursor molecule. FucTs are responsible for the final steps in this process. It has been demonstrated that the Le^x synthesis occurs similarly in *H. pylori* as observed in mammalian cells; L-fucose is added to the Type II precursor LacNAc by α1,3-FucTs (Figure 6) (Chan *et al*., 1995). *H. pylori* NCTC 11637 strain has two α1,3-*FucT* genes, *futA* and *futB*, encoding two α1,3-FucTs with different specificities. Appelmelk and colleagues (1999) have suggested that in the *H. pylori* NCTC 11637 strain, the *futA* gene product fucosylates internal LacNAc, while the *futB* gene product fucosylates the terminal LacNAc of O-antigen.

H. pylori has apparently developed unique biosynthetic pathways for the Le^y and Le^b antigens (Figure 6). In the synthesis of the Le^y antigen, the H type II antigen is a normal precursor in mammals, whereas the Le^x antigen is primarily used as a precursor in *H. pylori*. This is consistent with the observation that *H. pylori* strains are not known to express the H type II antigen (reviewed in Wang *et al*., 2000). However, the *H. pylori* α1,3-FucTs have been demonstrated *in vitro* to utilize both LacNAc and H Type II as acceptor molecules (Martin *et al*., 1997; Rasko *et al*., 2000). In the Le^b synthesis, the H Type II antigen is the normal biosynthetic precursor in mammals, whereas in *H. pylori* the Le^a antigen may be the primarily used precursor. The *H. pylori* α1,2-FucTs have been shown to utilize monofucosylated Lewis antigens (Le^x and Le^a) as acceptor molecules in the synthesis of difucosylated Lewis antigens (Le^y and Le^b). On the contrary, mammalian α1,2-FucTs do not normally utilize monofucosylated substrates. Furthermore, the *H. pylori* α1,2-FucTs have been demonstrated to utilize unfucosylated acceptor molecules such as the Le^c antigen and LacNAc leading to the formation of the H Type I and Type II antigens, respectively (Wang *et al*., 1999). However, as mentioned above, the *H. pylori* strains are not known to express the H Type II antigen. The characterized α1,3/1,4-FucT is capable of utilizing the Le^c and H Type I antigens as acceptors *in vitro*, although the 1,4-FucT activity with the H Type I antigen as a substrate is relatively weak (Rasko *et al*., 2000).

H. pylori and eukaryotic FucTs are highly divergent in overall amino acid sequences and in domain structures. All the mammalian fucosyltransferases have a domain structure containing a short N-terminal cytoplasmic tail, a transmembrane domain, and a stem region followed by a large globular C-terminal catalytic domain. The N-terminal cytoplasmic tail and transmembrane domain have a role in the Golgi localization and in the retention of the enzyme. In contrast, the amino acid sequences of *H. pylori* FucTs are devoid of a N-terminal transmembrane domain. The *H. pylori* α1,3 FucT has been shown to be membrane associated, whereas the α1,2 FucT has been found to be a soluble protein (reviewed in Wang *et al*., 2000). Oriol and collegues (1999) have suggested that eukaryotic and prokaryotic α1,2-, α1,3- and α1,6-fucosyltransferases might have a common ancestor or alternatively, α1,2/6-fucosyltransferases have one common ancestor and α1,3-fucosyltransferases have a

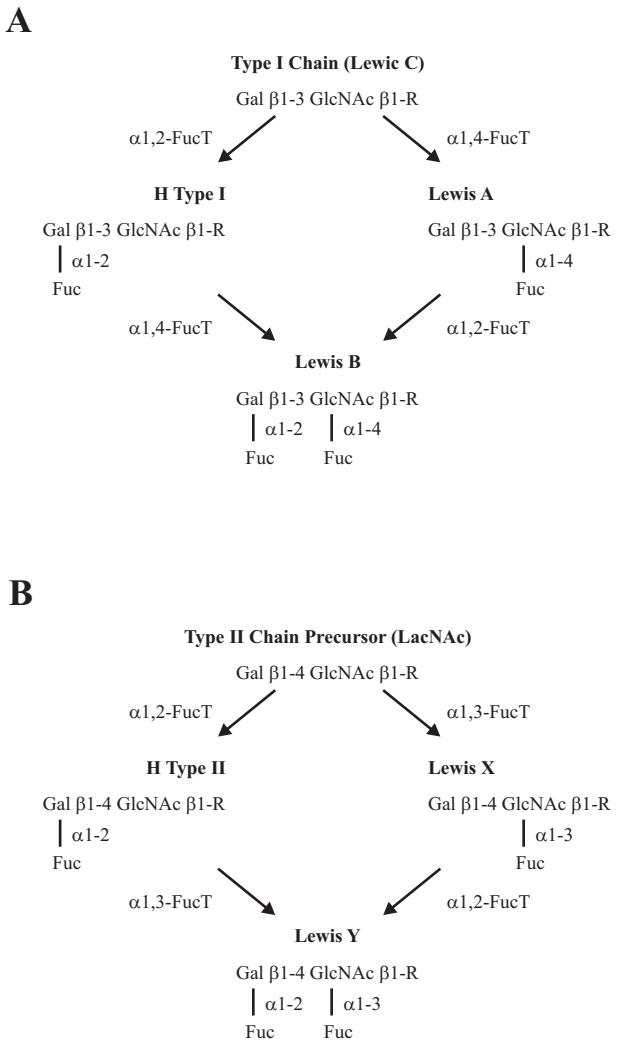

Figure 6. Biosynthesis of Lewis glycans. Panel A illustrates the biosynthesis of mono- and difucosylated Lewis antigens from the Type I precursor and panel B illustrates the synthesis from the Type II precursor. The only difference between the Type I and Type II precursors is the β1,3- and β1,4-linkages of the monosaccharides in the backbone glycans, respectively. FucT, fucosyltrasferase; Gal, galactose; Fuc, fucose; GlcNAc, *N*-acetyl-D-glucosamine; R, an acceptor molecule.

common ancestor of their own (Oriol *et al*., 1999). Furthermore, Breton and coworker (1998) have suggested that since all fucosyltransferases utilize GDP-L-fucose as a L-fucose donor, their specificity will probably reside in the recognition of the acceptor and in the type of linkage formed (Breton *et al*., 1998). Detailed studies on *H. pylori* (discussed above) and human FucTs (Niemelä *et al*., 1998; Toivonen *et al*., 2002) support this hypothesis.

α1,3/4-*fucTs* genes (also known as *futA/B*) of *H. pylori* contain characteristic polyadenine - polycytosine tracts at the 5´ end that have been identified as the cause of DNA slippage leading to the on/off switch of the target gene at the translational level (Appelmelk *et al*., 1999). The α1,3/4-*fucTs* genes also contain a 21-mer repeat region at the 3´ end that may play a role in the dimerisation and translational control of the FucT proteins (Ge *et al*., 1997). The α1,2-*fucT* gene (also named as *fut*C) contains similar polycytosine tract like the α1,3/4-*fucTs* genes as well as an adenine rich sequence at mid-region of the gene that may be responsible for slipped strand mispairing leading to the on/off status of α1,2-*fucT*. The nucleotide repeats in α1,2-*fucT* precede or belong to the sequence regulating the translational frameshifting that may also be responsible for the slipped strand mispairing (Wang *et al*., 1999). These *cis*-elements of *H. pylori fucT* genes lead to the phase variation of Lewis antigens. As mentioned above, the antigenic phase variation is most probably beneficial to *H. pylori* in adaptation to different environments and environmental changes, but its role in colonization and pathogenesis is unclear.

2.7. Genetic organization of genes related to L-fucose metabolism

In bacteria, the genes required for the synthesis of nucleotide sugars are generally scattered within the same gene cluster for a particular bacterial polysaccharide. GDP-L-fucose pathway genes have been reported to belong to the *wb* (formely named as *rfb*) gene cluster that encodes O-antigen synthesis (Zhang *et al*., 1996) or the CA gene cluster that encodes colanic acid synthesis (Stevenson *et al*., 2000). For example, in *E. coli* K12, GDP-L-fucose pathway genes are located in the *wca* gene cluster, which is responsible for the synthesis of the L-fucose containing EPS colanic acid (Andrianopoulos *et al*., 1998). It has been suggested that similarity in the three-dimensional structures of *E. coli* GMD and GMER with the observation that the corresponding genes are adjacent to each other on the bacterial genome might be due to a common ancestor (Somoza *et al*., 2000).

In *H. pylori*, O-antigen synthesizing genes are not clustered in one locus, as is the case in other bacteria. Instead, only *manC*, the D-mannose-6-phosphate isomerase/GDP-D-mannose pyrophosphorylase gene (also known as *rfbM*), *gmd* (also known as *rfbD*) and *gmer* (also known as *wbcJ*) genes are clustered and the other genes required for the O-antigen synthesis are distributed throughout the genome (Alm *et al*., 1999; Tomb *et al*., 1997). Berg and coworkers (1997) have suggested that this unusual genetic organization in *H. pylori* may be due to the special feature of *H. pylori* – host interaction that may favor the exchange of LPS synthesizing genes en bloc. Furthermore, the genetic organization is also in accordance with a proposed assembly mechanism of *H. pylori* O-antigen that differs from the mechanism used by other Gram-negative bacteria. Instead of transferring subunits consisting of several monosaccharides onto the growing O-antigen, the *H. pylori* O-antigen may be assembled by sequential addition of a single monosaccharide (Berg *et al*., 1997; Rasko *et al*., 2000).

3. D-RHAMNOSE PATHWAY

3.1. Prevalence of rhamnose

Rhamnose is a deoxyhexose sugar found widely in bacteria and plants, but not in humans. Of the two isomers, L and D, the former is much more common. L-rhamnose is found in a wide variety of both Gram-negative and Gram-positive bacteria, in which it is a common component of cell wall and capsule structures. The widespread prevalence of L-rhamnose and its relevance for several clinically significant pathogens has made the biosynthetic pathway of L-rhamnose an appealing target for novel therapeutic interventions. Humans neither synthesize nor utilize L-rhamnose (reviewed in Giraud and Naismith, 2000).

The activated nucleotide sugar form of L-rhamnose is dTDP-L-rhamnose, which is synthesized from glucose-1-phosphate and deoxythymidine triphosphate (dTTP) via a biosynthetic pathway requiring four enzymes (Figure 2). The first enzyme in the pathway is glucose-1-phosphate thymidylyltransferase (RmlA, EC 2.7.7.24), which catalyzes the transfer of a thymidylmonophosphate nucleotide to glucose-1-phosphate. The catalytic activity of RmlA is most likely regulated by the end product of the pathway, dTDP-L-rhamnose (Blankenfeldt *et al*., 2000). A similar specific feedback inhibitory mechanism has also been detected in the biosynthetic pathways of GDP-L-fucose (Sturla *et al*., 1997) and GDP-D-mannose (Wu *et al*., 2002).

The second enzyme dTDP-D-glucose 4,6-dehydratase (RmlB, EC 4.2.1.46) catalyzes an oxidation of the C4 hydroxyl group of the D-glucose residue that is followed by dehydratation, which leads to the formation of dTDP-4-keto-6-deoxy-D-glucose. Mechanistically, the catalytic action of RmlB is closely related to that of GMD (Allard *et al*., 2001; Allard *et al*., 2000; Somoza *et al*., 2000).

The third enzyme dTDP-4-keto-6-deoxy-D-glucose 3,5-epimerase (RmlC, EC 5.1.3.13) catalyzes a double epimerisation reaction at the C3 and C5 positions of the 4-keto-6-deoxy-D-glucose ring (Graninger *et al*., 1999). GMER also catalyzes a double epimerisation at positions C3 and C5 of the 4-keto-6-deoxy-D-mannose ring, but RmlC shows no structural similarity to GMER (Christendat *et al*., 2000; Giraud *et al*., 2000; Rosano *et al*., 2000). The cofactor (NADPH) and the sugar nucleotide binding sites of GMER have no counterpart in RmlC and thereby Giraud and colleagues (2000) have suggested that the mechanisms of epimerization are likely to be different in these enzymes.

Finally, in the synthesis of dTDP-L-rhamnose the fourth enzyme is dTDP-4-keto-6-deoxy-L-mannose reductase (RmlD, EC 1.1.1.133), which reduces the C4 keto group of the 4-keto-6-deoxy-L-mannose moiety and leads to the formation of dTDP-L-rhamnose (Graninger *et al*., 1999). RmlD shares sequence homology with GalE, GMD, and GMER in those amino acids known to have catalytic function (Blankenfeldt *et al*., 2002; Rosano *et al*., 2000; Somoza *et al*., 2000; Thoden *et al*., 1996). RmlD also shares amino acid homology with its direct analogue a GDP-4-keto-6-deoxy-D-mannose

reductase (RMD) involved in the synthesis of GDP-D-rhamnose (Kneidinger *et al*., 2001). The RmlA-D enzymes are highly conserved among microorganisms and their three-dimensional structures have been resolved (Allard *et al*., 2001; Allard *et al*., 2000; Blankenfeldt *et al*., 2000; Blankenfeldt *et al*., 2002; Christendat *et al*., 2000; Giraud *et al*., 2000). The genes encoding the RmlA-D enzymes are located in the *wb* (formely known as *rfb*) gene cluster together with genes required for the assembly of the particular bacterial polysaccharide. However, the four *rml* genes may not necessarily be clustered next to each other (reviewed in Giraud and Naismith, 2000).

D-rhamnose, the enantiomer of L-rhamnose, is not a very common 6-deoxyhexose. Hitherto, it has mainly been found as a constituent of EPS or LPS of Gram-negative bacteria, such as plant pathogens, *e.g. P. syringae* (Ovod *et al*., 1996) and *Xanthomonas campestris* (Molinaro *et al*., 2003) and human pathogens, *e.g. P. aeruginosa* (Yokota *et al*., 1987), *C. fetus* (Senchenkova *et al*., 1996), *H. pylori* (Kocharova *et al*., 2000), *Burkholderia cepacia* (Cerantola and Montrozier, 1997), and *Stenotrophomonas maltophilia* (Winn and Wilkinson, 1998). In *H. pylori*, the D-rhamnose residues containing O-antigen is atypical and it is only detected in some Danish strains, which are nontypeable with antibodies against Lewis and other blood group determinants (Kocharova *et al*., 2000). The biosynthesis of GDP-D-rhamnose (Figure 7) is described in detail in section 3.3.

3.2. D-rhamnose in *P. aeruginosa* LPS

P. aeruginosa is a natural soil inhabitant with a very versatile metabolic potential permitting it to be able to survive in a number of environments. It is also a human opportunistic pathogen infecting mainly immunocompromised patients and causing a range of severe infections. A new clinical problem is the rapidly increasing resistance of *P. aeruginosa* against commonly used antibacterial drugs (Kiska and Gilligan, 1999). Hence, the virulence factors and the pathogenesis of *P. aeruginosa* have recently gained much interest. *P. aeruginosa* LPS plays an important role in the virulence of this ubiquitous bacterium. *P. aeruginosa* synthesizes concomitantly two chemically and antigenically distinct forms of O-antigen, known as a common A-band and a serotype-specific B-band (reviewed in Rocchetta *et al*., 1999). The A-band O-antigen is a homopolymer consisting of D-rhamnose sugar residues arranged as repeating trisaccharide units (->3D-Rhaα1->2D-Rhaα1->3D-Rhaα1->)$_n$ (Arsenault *et al*., 1991). In contrast, the B-band O-antigen is a heteropolymer composed of repeating di- to pentasaccharide units of many different monosaccharides (Knirel and Kochetkov, 1994). The constitutively expressed A-band O-antigen is shorter and neutrally charged and, in the absence of the B-band LPS, confers hydrophobic properties upon *P. aeruginosa* (Arsenault *et al*., 1991; Lam *et al*., 1989). The B-band O-antigen is highly anionic and has more O-antigen repeat units than the A-band O-antigen thereby masking it (Lam *et al*., 1989). The B-band O-antigen has been shown to impede phagocytosis and is responsible for resistance to host serum (Engels *et al*., 1985). D-rhamnose is found only in the A-band O-antigen, while L-rhamnose is found in the core oligosaccharide (Rahim *et al*., 2000), B-band O-antigen and rhamnolipids of *P. aeruginosa* (reviewed in Maier and Soberon-Chavez, 2000; Rocchetta *et al*., 1999).

The gene clusters responsible for the synthesis of A- and B-band band LPS have been identified and their genetics and regulatory mechanisms as well as their relevance to the pathogenesis of *P. aeruginosa* are under extensive study. The availability of the whole genome sequence of *P. aeruginosa* has contributed to these studies (Stover *et al*., 2000). On the basis of mutagenesis studies, it has been proposed that in the A-band gene cluster the function of open reading frame (ORF) after the *gmd* gene could be involved in the synthesis of GDP-D-rhamnose (Rocchetta *et al*., 1998).

P. aeruginosa is commonly isolated from the specimens obtained from the lungs of patients suffering the congenital monogenic disease cystic fibrosis (CF). The respiratory *P. aeruginosa* isolates from the chronic CF infection are devoid of O-antigen or mainly express the D-rhamnosylated A-band O-antigen (reviewed in Lyczak *et al*., 2002; Rocchetta *et al*., 1999). Hitherto, no specific condition has been identified to influence the synthesis or regulation of A-band LPS. Interestingly, exactly the same D-rhamnan polysaccharide structure as observed in the A-band LPS of *P. aeruginosa* has also been found from the other opportunistic pathogens *B. cepacia* and *S. maltophilia* also associated with the CF disease with severe pulmonary manifestation (Cerantola and Montrozier, 1997; LiPuma, 2000; Winn and Wilkinson, 1998). In addition, *B. cepacia* and *S. maltophilia* have also emerged as important multidrug resistant pathogens and a cause of nosocomial infections (reviewed in Mahenthiralingam *et al*., 2002; Zhang *et al*., 2000).

3.3. GDP-D-rhamnose metabolism

Markovitz proposed a biosynthetic pathway for GDP-D-rhamnose in the 1960s (Markovitz, 1964). Recently, Kneidinger and colleagues (2001) provided evidence for the proposal by cloning the enzymes required for the synthesis of GDP-D-rhamnose. The GDP-D-rhamnose pathway starts from GDP-D-mannose (Figure 7), which is converted into GDP-4-keto-6-deoxy-D-mannose by GMD, as is the case in the synthesis of GDP-L-fucose (Figure 3). The following step in the GDP-D-rhamnose pathway is the specific reduction of the 4-keto group of GDP-4-keto-6-deoxy-D-mannose, which leads to formation of GDP-D-rhamnose, and which is catalyzed by GDP-4-keto-6-deoxy-D-mannose reductase (RMD) (EC 1.1.1.187). During the action of reduction RMD can utilize either NADH or NADPH as hydride donors. The reduction activity of RMD is analogous to the activity of the RmlD enzyme, which is responsible for the reduction of the C4 keto group of dTDP-4-keto-6-deoxy-L-mannose in the synthesis of dTDP-L-rhamnose (Giraud *et al*., 1999). Kneidinger and colleagues (2001) identified two enzymes, GMD and RMD, responsible for the GDP-4-keto-6-deoxy-D-mannose reductase activity from the non-pathogenic, Gram-positive *A. thermoaerophilus* L420-91^T bacterium. D-rhamnose is a constituent of the surface layer glycoprotein of this bacterium. The specific function of D-rhamnosylated S-layer of *A. thermoaerophilus* L420-91^T is unknown.

3.4. GDP-4-keto-6-deoxy-D-mannose reductase

A. thermoaerophilus GMD has been shown to be a bifunctional enzyme with both GDP-mannose 4,6-dehydratase and GDP-4-keto-6-deoxy-D-mannose reductase

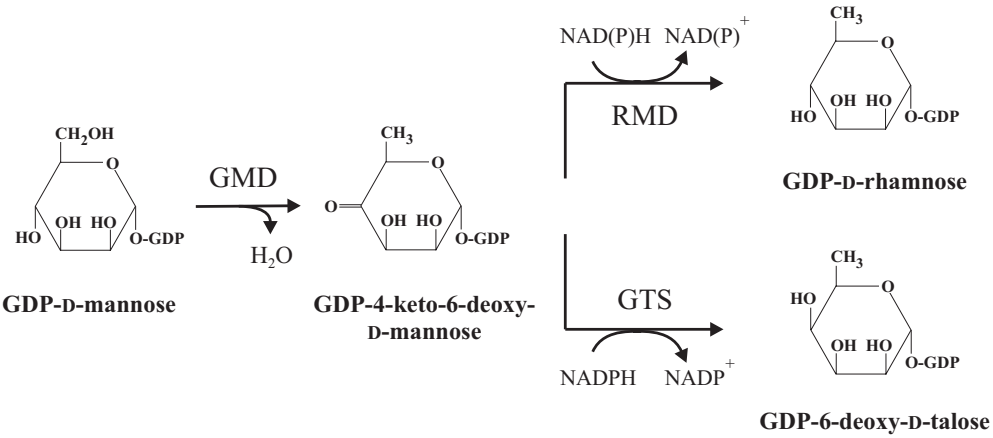

Figure 7. Schematic presentation of the biosynthetic pathways of GDP-D-rhamnose and GDP-6-deoxy-D-talose. GMD, GDP-D-mannose 4,6-dehydratase; RMD, GDP-4-keto-6-deoxy-D-mannose reductase; GTS, GDP-6-deoxy-D-talose synthetase; GDP, guanosine diphosphate.

activity, however the latter activity is relatively weak in comparison to the activity of *A. thermoaerophilus* RMD acting only as a reductase (Kneidinger *et al*., 2001). The amino acid residues reported to be important for the catalysis of the dehydratase reaction or for the binding of NADP cofactor or GDP-D-mannose substrate in *E. coli* GMD (Somoza *et al*., 2000) are also found in *A. thermoaerophilus* GMD, with the exception that in the catalytic triad (Thr-Tyr-Lys) Thr replaces Ser. The catalytic triad Ser-Tyr-Lys is more common among the enzymes belonging to the SDR protein family than a triad consisting of Thr-Tyr-Lys (Jörnvall *et al*., 1995), which is hitherto observed only in GMDs (Somoza *et al*., 2000). The amino acid sequence GILFNHES, which appears to be characteristic of GMDs (Somoza *et al*., 2000), is also found in *A. thermoaerophilus* GMD. The molecular mass of the monomer of *A. thermoaerophilus* GMD is *ca*. 37 kDa, whereas possible multimerisation of the enzyme is unknown. To date, *A. thermoaerophilus* GMD is the only enzyme identified to catalyze both dehydratase and reductase reactions. A possible explanation for the bifunctional nature of *A. thermoaerophilus* GMD could be mutations in the *gmd* gene (Kneidinger *et al*., 2001).

Genes encoding enzymes involved in the synthesis of nucleotide sugars are usually found in the gene cluster for the particular bacterial polysaccharide. In the genome of *A. thermoaerophilus, rmd* is located directly downstream of *gmd*. The characterized *A. thermoaerophilus* RMD acts only as a GDP-4-keto-6-deoxy-D-mannose reductase in the synthesis of GDP-D-rhamnose. The molecular mass of the monomer of RMD is *ca*. 35 kDa, whereas the possible multimerisation of the enzyme is unknown. The amino acid sequence of RMD contains an N-terminal coenzyme-binding pattern GlyXXGlyXXGly, and an active-site Ser-Tyr-Lys triad common to the SDR protein family. Other residues involved in GMD catalysis (Somoza *et al*., 2000), with the exception of Glu135 are not found in RMD (Kneidinger *et al*., 2001). In GMD catalysis,

Glu135 is proposed to play a role in the deprotonation/protonation of the hydroxyl group of the C5 position of GDP-4-keto-6-deoxy-D-mannose (Somoza *et al*., 2000), but this C5 atom is not involved in the reduction reaction catalyzed by RMD (Kneidinger *et al*., 2001).

3.5. Rhamnosyltransferases

GDP-D-rhamnose and dTDP-L-rhamnose are used as substrates for specific rhamnosyltransferases that are responsible for transferring L-/D-rhamnose to an acceptor molecule via a specific linkage. Currently, very little is known about the bacterial rhamnosyltransferases. L-rhamnosyltransferases, which use dTDP-L-rhamnose as a donor, have been reported in several bacteria (reviewed in Giraud and Naismith, 2000), whereas putative D-rhamnosyltransferases, which use GDP-D-rhamnose as a donor, have only been identified from *P. aeruginosa* (Rocchetta *et al*., 1998). On the basis of mutagenesis studies it has been shown that three putative D-rhamnosyltransferases (WbpZ, WbpY and WbpX) participate in the synthesis of the linear A-band O-antigen in *P. aeruginosa*. Specific functions or detailed acceptor specificities of WbpZ, WbpY and WbpX have not been experimentally verified. However, Rocchetta and coworkers (1998) have proposed that in the synthesis of repeating D-rhamnan structure (->3D-Rhaα1->2D-Rhaα1->3D-Rhaα1->)$_n$ WbpZ may be responsible for transferring the first D-rhamnose moiety to the acceptor molecule via an α1->3 linkage. The initial A-band O-antigen acceptor molecule is probably L-rhamnose, which is also the initial B-band O-antigen acceptor molecule (Rahim *et al*., 2000). WbpY may be responsible for transferring the following two α1->3 linked D-rhamnose moieties to the first D-rhamnose. The terminal α1->3 linked D-rhamnose would then act as an acceptor for WbpX, which adds one D-rhamnose moiety to the A-band polymer via an α1->2 linkage. Both WbpY and WbpX would then continue to synthesize the A-band O-antigen assuming that WbpY recognizes dual acceptors, the first α1->3 linked D-rhamnose transferred by WbpZ and the α1->2 linked D-rhamnose transferred by WbpX. This hypothesis is consistent with the genetic organization of the *wbpX*, *wbpY* and *wbpZ* genes, which are located in the opposite order to which the encoded enzymes act. A similar genetic arrangement coupled to the sequential order of the O-antigen assembly has been seen with other glycosyltransferases (Kido *et al*., 1995).

4. 6-DEOXY-D-TALOSE PATHWAY

4.1. Prevalence of 6-deoxy-talose

6-deoxy-talose is a rare deoxyhexose sugar. To date, it is found only in bacteria. Of the two isomers, L and D, the former is more common. 6-deoxy-L-talose is found in a few Gram-negative and Gram-positive bacteria, in which it is a constituent of cell wall and capsule structures. The activated nucleotide sugar form of 6-deoxy-L-talose is dTDP-6-deoxy-L-talose, which is synthesized from glucose-1-phosphate and dTTP via a biosynthetic pathway requiring four enzymes (Figure 2). The first three enzymes RmlA-C are the same as in the biosynthetic pathway of dTDP-L-rhamnose. The fourth enzyme Tll is also a dTDP-4-keto-6-deoxy-L-mannose reductase like RmlD, but the stereoselectivity of Tll determines that dTDP-4-keto-6-deoxy-L-mannose is reduced to dTDP-6-deoxy-L-talose (Nakano *et al*., 2000).

6-deoxy-D-talose is an enantiomer of 6-deoxy-L-talose. GDP-6-deoxy-D-talose is an activated nucleotide sugar form of 6-deoxy-D-talose, which has been found only in EPS or LPS structures of a limited number of Gram-negative bacteria, such as *A. actinomycetemcomitans* serotype a and *Burkholderia* (*Pseudomonas*) *plantarii* strain DSM 6535 (Shibuya *et al*., 1991; Weckesser *et al*., 1973; Zähringer *et al*., 1997). The biosynthesis of GDP-6-deoxy-D-talose (Figure 7) is decribed in detail in section 4.3.

4.2. 6-deoxy-D-talose in *A. actinomycetemcomitans* LPS

An *O*-acetylated, linear homopolysaccharide of 6-deoxy-D-talose has been found in the serotype a-specific polysaccharide antigen (SPA) of *A. actinomycetemcomitans* (Perry *et al*., 1996; Shibuya *et al*., 1991) and the EPS of the *B. plantarii* strain DSM 6535 (Zähringer *et al*., 1997). The 6-deoxy-D-talan polymer of *A. actinomycetemcomitans* serotype a is composed of repeating disaccharide units (->3 6dTalα1->2 6dTalα1->)$_n$, whereas 6-deoxy-D-talan of *B. plantarii* strain DSM 6535 is composed of repeating trisaccharide units (->3 6dTalα1->2 6dTalα1->2 6dTalα1->)$_n$. Both 6-deoxy-D-talan polymers are acetylated at the O2 position of α1->3 linked 6-deoxy-D-talose. The specific roles of 6-deoxy-D-talan O-polysaccharide (O-PS) of human opportunistic pathogen *A. actinomycetemcomitans* serotype a and 6-deoxy-D-talan EPS of plant pathogen *B. plantarii* strain DSM 6535 are unknown. In general, bacterial extracellular polysaccharides consisting of only one type of 6-deoxyhexose are rare. D-rhamnans and L-rhamnans are found in several bacteria, *e.g. P. aeruginosa* (Arsenault *et al*., 1991) and *Bacillus stearothermophilus* strain NRS 2004/3a (Christian *et al*., 1986), respectively. 6-deoxy-L-talans are found in the SPA of *A. actinomycetemcomitans* serotype c (Perry *et al*., 1996) and *Rhizobium loti* NZP2213 (Russa *et al*., 1995), and homopolymers of 6-deoxy-L-altrose are found in *Y. enterocolitica* O:3 (Hoffman *et al*., 1980) and *Pectinatus frisingensis* (Senchenkova *et al*., 1995).

In addition to 6-deoxy-D-talose and 6-deoxy-L-talose, other deoxyhexoses such as L-rhamnose and D-fucose are found in different SPAs of *A. actinomycetemcomitans*

(Amano *et al*., 1989; Perry *et al*., 1996). *A. actinomycetemcomitans* is a coccobacillus that colonizes the human oral cavity and has been implicated in the etiology of localized juvenile periodontitis (LJP), adult periodontitis and severe nonoral infections (reviewed in Henderson *et al*., 2002). The serologic specificity of *A. actinomycetemcomitans* strains is defined by 6 structurally and antigenically distinct O-PS components of their respective LPS (Gmur *et al*., 1993; Kaplan *et al*., 2001; Saarela *et al*., 1992). These serotype specific polysaccharides (a-f) constitute the outermost surface of the bacterium and have thus been suggested to play a role in the virulence of *A. actinomycetemcomitans* (reviewed in Fives-Taylor *et al*., 2000). 6-deoxy-D-talan containing *A. actinomycetemcomitans* serotype a strain has been isolated from healthy subjects as well as patients with LJP or adult perodontitis (Zambon *et al*., 1983). The gene cluster associated with the biosynthesis of 6-deoxy-D-talan SPA of *A. actinomycetemcomitans* SUNYaB 75 has been identified (Suzuki *et al*., 2000) and it contains 14 ORFs, 9 of which were not found in SPA-associated gene clusters of the other serotypes. Furthermore, the serotype a-SPA associated gene cluster is located in the different region of the chromosome than the other SPA associated gene clusters (Kaplan *et al*., 2001).

4.3. GDP-6-deoxy-D-talose metabolism

Markovitz proposed the biosynthetic pathway of GDP-6-deoxy-D-talose in 1960s, although the corresponding enzymes were not specifically identified (Markovitz, 1964). Like the other two GDP-6-deoxyhexose pathways introduced above in sections 2.3 and 3.3, the GDP-6-deoxy-D-talose pathway starts from GDP-D-mannose, which is converted into GDP-4-keto-6-deoxy-D-mannose by GMD (Figure 7). The following step in the GDP-6-deoxy-D-talose pathway is the specific reduction of the 4-keto group of GDP-4-keto-6-deoxy-D-mannose, which leads to the formation of GDP-6-deoxy-D-talose. 6-deoxy-D-talose is an epimer of D-rhamnose and differs from it only in the orientation of the hydroxyl group at the C4 position (Figure 7). The stereoselectivity of GDP-4-keto-6-deoxy-D-mannose reductase determines which one of these two epimers is synthesized. The characterization of GDP-4-keto-6-deoxy-D-mannose reductase responsible for the synthesis of GDP-6-deoxy-D-talose is described in this study. Other enzymes related to talosylation, *e.g.* deoxytalosyltransferases, as well as the regulatory mechanisms of talosylation remain to be elucidated.

5. AIMS OF THE STUDY

Bacterial cell surface polysaccharides have important roles in the interplay between environment and bacteria, especially in interplay between host and pathogen. The biosynthesis of cell surface glycoconjugates involves specific glycosyltransferases utilizing nucleotide sugars as activated donors. The alteration of cellular nucleotide sugar levels enables manipulation of bacterial cell surface polysaccharides. Hence, enzymes involved only in the biosynthesis of bacterial nucleotide sugars could be considered as potential targets for antimicrobial therapeutics. However, more detailed studies are needed to fully understand the enzymology related to bacterial nucleotide sugar metabolism.

Nucleotide sugars GDP-L-fucose, GDP-D-rhamnose, and GDP-6-deoxy-D-talose are converted from GDP-D-mannose by specific enzymes. The principal goals of this study were:

- to clone and characterize bacterial enzymes involved in the conversion of GDP-D-mannose into GDP-deoxyhexoses
- to develop of an ion-pair reversed-phase HPLC method for the study of synthesized nucleotide sugars

6. MATERIALS AND METHODS

Bacterial and yeast strains and plasmids used in this study are listed in Table 3 and 4. The methods used in this study are described in detail in the indicated articles and are summarized in Table 5.

Table 3. Bacterial and yeast strains used in this study

Strain	Characteristics[a]	Article	Reference
E. coli XL1Blue		I	
E. coli TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX*74 *deoR recA1* *araD*139 Δ(*ara-leu*)7697 *galU galK* *rpsL*(Str^R) *endA1 nupG*	II, IV	Invitrogen,Inc.
E. coli BL21(DE3)	B F$^-$ *dcm ompT hsdS*(r_B^- m_B^-) *gal*	IV	
H. pylori NCTC 11637		I	ATCC 43504
P. aeruginosa		II, III	ATCC 27853
A. actinomycetemcomitans	Serotype a	IV	ATCC 29523
S. cerevisiae YPH501	*ura*3-52 *lys*2-801^{amber} *ade*2-101^{ochre} *trp*1--Δ63 *his*3-Δ200 *leu*2-Δ1, mating type a/α	I, II, III	Invitrogen,Inc.

[a](Sambrook and Russell, 2001)

Table 4. Plasmids used in this study

Plasmid	Relevant property	Reference
pESC-LEU	*S. cerevisiae* expression vector	Stratagene,Inc.
pHP1	pESC-LEU derivate containing the *H. pylori gmd* gene as a 1143 bp fragment under the GAL10 promoter	I, II, III
pHP2	pESC-LEU derivate containing the *H. pylori gmer* gene as a 952 bp fragment under the GAL10 promoter	I
pHP3	pESC-LEU derivate containing the *H. pylori gmd* gene under the GAL10 promoter and the *H. pylori gmer* gene under the GAL10 promoter	I, III
pRHA1	pESC-LEU derivate containing the *P. aeruginosa rmd* gene as a 915 bp fragment under the GAL1 promoter	II
pRHA2	pESC-LEU derivate containing the *H. pylori gmd* gene under the GAL10 promoter and the *P. aeruginosa rmd* gene under the GAL1 promoter	II, III
pCR-XL-TOPO	*E. coli* cloning vector	Invitrogen, Inc.
pASK-IBA5	*E. coli* expression vector	IBA, GmbH.
pKETO	pASK-IBA5 derivate containing the *H. pylori gmd* gene as a 1143 bp fragment under the *tet* promoter	IV
pTAL	pASK-IBA5 derivate containing the *A. actinomycetemcomitans gts* gene as a 885 bp fragment under the *tet* promoter	IV

Table 5. Methods used in this study

Method	Described and used in
Culture conditions	
H. pylori	I
P. aeruginosa	II
A. actinomycetemcomitans	IV
S. cerevisiae	I, II
Genetic methods	
Isolation of chromosomal DNA	I, II, IV
Molecular cloning techniques	I, II, IV
Sequence analysis	I, II
Protein methods	
Expression of fusion proteins	I, II, IV
Affinity chromatography	IV
Sequence analysis	I, II, IV
Immunological methods	
Western blot	I, II
Enzyme assays	
Fucosyltransferase assay	I
Enzymatic reactions	I, II, III, IV
Methods of carbohydrate chemistry	
DEAE anion exchange	I, II, III
Solid-phase extraction	III, IV
Ion-pair reversed-phase HPLC	I, II, III, IV
MALDI-TOF Mass spectrometry	II, III, IV
NMR	II, III, IV

7. RESULTS AND DISCUSSION

7.1. *H. pylori* GDP-L-fucose synthesizing enzymes GMD and GMER (I)

7.1.1. Functional cloning of GMD and GMER (I)

H. pylori ORFs of GMD and GMER were identified by the amino acid sequence similarities with the corresponding *E. coli* enzymes and by the annotations of the whole genome sequences of *H. pylori* 26695 (Tomb *et al*., 1997) and J99 (Alm *et al*., 1999). In the genomic databases of *H. pylori*, HP0044 ORF was annotated as putative GMD and HP0045 ORF was annotated as putative nodulation protein or GMER. The identified *gmd* and *gmer* genes were amplified from the *H. pylori* NCTC 11637 chromosomal DNA. The 1143 base pairs (bp) *gmd* gene was inserted under the galactose inducible GAL1 promoter of the yeast expression vector pESC-LEU in-frame with the N-terminal c-myc epitope. And the 952 bp *gmer* gene was inserted under the galactose inducible GAL10 promoter of the pESC-LEU vector in-frame with the N-terminal FLAG epitope. Furthermore, a construct containing both genes with the same N-terminal epitopes as above was prepared. Resulting plasmids pHP1, pHP2 and pHP3, respectively, were used for overexpression of the fusion proteins in *S. cerevisiae* YPH501 (Table 1). The heterologous expression of GMD and GMER was studied by Western blotting with antibodies against the N-terminal epitopes.

The expression of *ca*. 44 kDa c-myc tagged GMD was detected with the c-myc antibody from the lysates of the yeast strains harboring either the *gmd* gene containing pHP1 plasmid or the *gmd* and *gmer* genes containing pHP3 plasmid (Figure 3 of article I, lanes 2 and 3). The expression of *ca.* 35 kDa FLAG tagged GMER was detected with the FLAG antibody from the lysates of the yeast strains harboring either the *gmer* gene containing pHP2 plasmid or the pHP3 plasmid (Figure 3/I, lanes 2 and 3). The apparent sizes of recombinant GMD and GMER corresponded to those calculated from the amino acid sequences. In addition to GMD and GMER, several other bands were detected in the Western blots probably due to degradation or different glycosylation of the recombinant proteins. However, corresponding bands were not detected from the vector control (Figure 3/I, lane 4).

7.1.2. Enzymatic activities of recombinant GMD and GMER (I)

In order to analyze functional activities of heterologously expressed GMD and GMER, a thorough analysis of the cellular sugar nucleotide pool of the recombinant yeast strains was performed. GMD was expected to utilize endogenous GDP-D-mannose of yeast as a substrate. Synthesized nucleotide sugars were analyzed by ion-pair reversed phase high-pressure liquid chromatography (HPLC) and by fucosyltransferase assay developed in our laboratory (III, Räbinä *et al*., 2000). In HPLC analysis, the cellular GDP-sugars were identified based on the retention times of commercially available GDP-sugar standards. Prior to HPLC analysis, the yeast lysates were purified with solid-phase extraction (SPE) and treated with alkaline phosphatase in order to avoid

nucleotide peaks in the HPLC chromatograms. The methodological development of this new sample preparation technique and ion-pair reversed-phase HPLC for the analysis of nucleotide sugars is described in detail in section 7.3 (III).

The HPLC analysis (Figure 4/I) showed that the lysate of *S. cerevisiae* YPH501(pHP3) strain co-expressing GMD and GMER gave a peak with the same retention time as the commercial GDP-L-fucose standard (Figure 4/I, panel D). This peak was not detected from the cytoplasmic extracts of the vector control strain or yeast strains expressing only one of these two enzymes (Figure 4/I, panels A-C). Instead, a novel peak with different retention time as GDP-L-fucose was detected from the lysate of *S. cerevisiae* YPH501(pHP1) strain expressing only GMD (Figure 4/I, panel B). This peak represented the intermediate product GDP-4-keto-6-deoxy-D-mannose. At the time of the experiment, this data could not be confirmed because of the lack of an available standard for this GDP-sugar. But with the present knowledge and available standard isolated from the cytoplasmic extract of *P. aeruginosa*, this data has been confirmed. In addition to these novel peaks that indicated the functional activities of GMD and GMER, some uncharacterized peaks from yeast cells were detected (Figure 4/I).

The quantity of GDP-L-fucose produced by the recombinant yeast strains was studied with a functional fucosyltransferase assay, in which an α1,3-fucosylated glycan is synthesized in the presence of human α1,3-fucosyltransferase VI and the fucose donor present in the recombinant yeast strain (Figure 5/I). In this assay, the limiting factor in the generation of fucosylated glycan is the presence or absence of GDP-L-fucose because the relevant acceptor molecule and transferase enzyme are always present. The detection of synthesized fucosylated glycans is based on specific antibodies and time-resolved immunofluorometry (Räbinä *et al.*, 2000). The results of this assay were consistent with the results obtained from HPLC analysis (Figure 4/I). Only the *S. cerevisiae* YPH501(pHP3) strain expressing both GMD and GMER was able to synthesize GDP-L-fucose and could be used as a source of GDP-β-L-fucose (Figure 5/I, panel B). The amount of GDP-L-fucose synthesized by the *S. cerevisiae* YPH501(pHP3) strain was determined to be 5 mmol/ml of the yeast culture (Figure 5/I, panel A).

The functional activities of *H. pylori* GMD and GMER were also confirmed by the independent cloning and characterization of HP0044 and HP0045 ORFs of *H. pylori* 26695 by Wu and colleagues (2001). Mutagenesis studies and analysis of whole genome sequences of *H. pylori* have shown that *gmd* and *gmer* are present as a single copy in the genome thus being responsible for all fucosylation in the bacterium (Alm *et al.*, 1999; McGowan *et al.*, 1998; Tomb *et al.*, 1997). The amino acid sequences of GMD and GMER contained the characteristics of the SDR protein family (Jörnvall *et al.*, 1995) and the proposed catalytic triad of GMD contained the amino acids Thr-Tyr-Lys like most of GMDs (Somoza *et al.*, 2000).

7.1.3. Synthesis of GDP-L-fucose in *S. cerevisiae* expression system (I)

In addition to the characterization of enzymatic activities of *H. pylori* GMD and GMER, this study showed that the *S. cerevisiae* expression system is applicable to the functional expression of bacterial genes synthesizing GDP-L-fucose from yeast endogenous GDP-D-mannose. In our laboratory, *E. coli* GMD and GMER have also been expressed functionally in the same expression system (Mattila *et al*., 2000). The concentrations of yeast endogenous GDP-D-mannose, $NADP^+$ and NADPH seem apparently to be adequate for the relatively effective production of GDP-L-fucose by recombinant bacterial enzymes. This offers a major advantage over other expression systems as no exogenous expensive GDP-D-mannose or cofactors need to be added to the reaction mixture with GMD and GMER. Furthermore, *S. cerevisiae* appears to be an ideal host for expressing enzymes needed for the synthesis of the deoxyhexose sugar nucleotides because its glycosylation is largely restricted to mannosylation and it is not known to have deoxyhexose metabolism of its own (Hashimoto *et al*., 1997; reviewed in Romanos *et al*., 1992). The quantity of GDP-L-fucose synthesized by recombinant *H. pylori* GMD and GMER was equivalent to the quantity obtained in the corresponding recombinant enzymes of *E. coli* (Mattila *et al*., 2000). Mattila and colleagues (2000) also showed that in this expression system the concentration of endogenous yeast GDP-D-mannose was the limiting factor for the synthesis of GDP-L-fucose. In yeast cells, GDP-D-mannose is synthesized in the cytoplasm and then transported to the Golgi apparatus (Abe *et al*., 1999). Hence, as an expression host it might be beneficial to use a mutant yeast strain, which has a defect in its GDP-D-mannose transportation system. Defects in this transportation system might lead to an accumulation of sytosolic GDP-D-mannose thus providing increased substrate concentration for GMD. However, the synthesis of GDP-D-mannose may be regulated by a similar feedback inhibitory mechanism as detected in the case of other nucleotide sugars, *e.g.* GDP-L-fucose and dTDP-L-rhamnose (Sturla *et al.,* 1997; Blankenfeldt *et al.*, 2000) or in the synthesis of GDP-D-mannose in *H. pylori* (Wu *et al*., 2002). The feedback inhibition mechanism might prevent the accumulation of sytosolic GDP-D-mannose in abundance.

7.1.4. Conservation of GMD and GMER homologs among bacteria (I)

The synthesis of GDP-L-fucose requires the activity of both *gmd* and *gmer* genes. The nucleotide and protein databases were searched against the sequence similarities of known GMDs and GMERs. At the time of the experiment, the number of bacteria having both *gmd* and *gmer* homologues was found to be relatively low (Table 1/I). Among them were *H. pylori*, *E. coli, Y. enterocolitica, R. fredii, Salmonella typhimurium, Vibrio cholerae,* and *Mycobacterium tuberculosis*. It was thought that this might indicate that unlike among higher eukaryotes, the ability of bacteria to fucosylate their glycans is not a very common feature. On the other hand, the number of bacteria having both *gmd* and *gmer* homologues may increase as the number of completely sequenced microbial genomes multiplies, for instance at present in addition to the bacteria listed above both *gmd* and *gmer* homologues can be found in *Y. pestis, S. enterica* serovar Typhi CT18 and *Shigella flexneri* 2a (Jin *et al*., 2002; Parkhill *et*

al., 2001; Parkhill *et al*., 2001). Hence, the ability of bacteria to fucosylate their glycans might indeed be a common feature and not be as rare as thought above. The phylogenetic analysis of the found bacterial *gmd* and *gmer* genes (Table 1/I) showed that the overall identity and similarity within the sequences of the *gmer* gene family was clearly lower (25% – 56%) in comparison to the *gmd* gene family (45% – 75%).

7.2. *P. aeruginosa* GDP-D-rhamnose synthesizing enzyme RMD (II)

7.2.1. Functional expression of RMD (II)

The complete *rmd* gene was identified from the A-band gene cluster of *P. aeruginosa* by sequence analysis tools available in the GCG package (Wisconsin Package, version 10.0; Genetics Computer Group, Madison, Wisconsin, USA) and by the annotation of PA5454 ORF of the whole genome sequence of *P. aeruginosa* (Stover *et al*., 2000). The size of the *rmd* gene was 915 bp and the starting codon was found to be TTG. Mutagenesis studies and analysis of whole genome sequences of *P. aeruginosa* have shown that *rmd* is present as a single copy in the genome thus it is responsible for all D-rhamnosylation in the bacterium (Rocchetta *et al*., 1998; Stover *et al*., 2000). The *rmd* gene was amplified from the *P. aeruginosa* chromosomal DNA and subcloned under the GAL10 promoter of the pESC-LEU vector in-frame with the N-terminal FLAG epitope. Because the characterized *H. pylori* GMD was needed to generate the GDP-4-keto-6-deoxy-D-mannose substrate for RMD, *rmd* was also subcloned into the same site of pHP1 construct, which is a pESC-LEU derivate containing *H. pylori gmd* (I). The resulting plasmids pRHA1 and pRHA2, respectively, and the pHP1 construct were used for overexpression of the fusion proteins in *S. cerevisiae* YPH501 (Table 1). The heterologous expression of GMD and RMD was studied by Western blotting with antibodies against the N-terminal epitopes.

The expression of *ca*. 44 kDa c-myc tagged GMD was detected with the c-myc antibody from the lysates of yeast strains harboring either the *gmd* gene containing pHP1 plasmid or the *gmd* and *rmd* genes containing pRHA2 plasmid (Figure 3/II, lanes 2 and 4). The expression of *ca.* 34 kDa FLAG tagged RMD was detected with the FLAG antibody from the yeast lysates harboring the *rmd* gene containing pRHA1 plasmid or the pRHA2 plasmid (Figure 3/II, lanes 3 and 4). The apparent size of recombinant RMD corresponded to that calculated from the amino acid sequences. In addition to GMD and RMD, several other bands were detected in the Western blots, as was the case with the Western blots of GMD and GMER in the previous study (Figure 3/I). These bands were probably due to degradation or different glycosylation of GMD and RMD. However, corresponding bands were not detected from the vector control (Figure 3/II, lane 1).

7.2.2. Enzymatic activity of recombinant RMD (II)

To analyze functional activities of heterologously expressed GMD and RMD, a thorough analysis of sugar nucleotides formed in reactions of recombinant *S. cerevisiae*

lysates with exogenously added GDP-D-mannose and $NADP^+$ and NADPH was performed. The sugar nucleotides from yeast lysates were analyzed with ion-pair reversed-phase HPLC (III), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and ^1H nuclear magnetic resonance (NMR).

The HPLC analysis (Figure 4/II) showed that the lysate of *S. cerevisiae* YPH501(pHP1) strain expressing GMD gave a similar peak profile containing GDP-D-mannose and GDP-4-keto-6-deoxy-D-mannose (Figure 4/II, panel C) as seen in the HPLC profile of the same strain in Figure 4/I, panel B. As no standard was available for the GDP-4-keto-6-deoxy-D-mannose intermediate product, the presence of GDP-4-keto-6-deoxy-D-mannose in reaction mixture was studied by chemical reduction with $NaBH_4$. After chemical reduction, two new peaks, GDP-D-rhamnose and GDP-6-deoxy-D-talose, were detected with HPLC (data not shown). At the time of the experiment, the GDP-6-deoxy-D-talose peak could not be confirmed because of the lack of an available standard for this GDP-sugar. However, with the present knowledge and the available standard for this GDP-sugar (IV), this data has been confirmed. The GDP-4-keto-6-D-deoxy-mannose peak was not detected from the lysate of the *S. cerevisiae* YPH501(pRHA2) strain co-expressing GMD and RMD. Instead, a novel peak with a retention time different from that of GDP-4-keto-6-deoxy-D-mannose or any other GDP-sugar standard was detected (Figure 4/II, panel D). This peak was further analyzed by MALDI-TOF MS. MALDI-TOF MS analysis gave a single peak at *m/z* 588.05, which is the mass of the GDP-deoxyhexoses (calculated *m/z* for $[M-H]^-$ is 588.08). The novel GDP-deoxyhexose peak together with the GDP-4-keto-6-deoxy-D-mannose peak was also seen when the lysates of yeast strains expressing either GMD or RMD were mixed together (Figure 4/II, panel E). The peaks of interest were not detected from the HPLC chromatograms of the vector control strain or the yeast strain expressing RMD (Figure 4/II, panel A-B). In addition to the peaks that indicated the functional activities of GMD and RMD, some uncharacterized peaks from yeast cells were detected (Figure 4/I). The addition of NADPH as a cofactor was required for the RMD activity.

To establish the structure of the novel GDP-deoxyhexose, it was analyzed by ^1H NMR spectroscopy. The ^1H NMR spectrum of the novel GDP-deoxyhexose was assigned (Figure 5/II) and the proton-proton coupling constants $^3J_{H,H}$ (Table 2/II) were determined from a DQFCOSY spectrum (data not shown). The NMR results established the structure as GDP-rhamnose with the characteristic presence of a methyl group bound to the carbon atom 5. The coupling constants between the ring protons of the rhamnosyl unit were characteristic of a mannoconfiguration and clearly distinguished the structure from GDP-L-fucose. The chemical shifts obtained were similar to those published by Kneidinger and colleagues (2001) for GDP-D-rhamnose and different from those reported for GDP-L-fucose (Adelhorst and Whitesides, 1993).

7.2.3. Synthesis of GDP-D-rhamnose in *S. cerevisiae* expression system (II)

The yield of synthesized GDP-D-rhamnose after the purification steps was determined with HPLC. As calculated from GDP-D-mannose added to the cell lysates, 3-4% of

the substrate was converted into GDP-D-rhamnose with the lysate of the *S. cerevisiae* YPH501(pRHA2) strain. The yield of GDP-D-rhamnose was 9% in a sample in which the lysates of yeast strains expressing either GMD or RMD were mixed together. In the *S. cerevisiae* expression system, the concentration of endogenous GDP-D-mannose has been shown to be the limiting factor for the synthesis of GDP-L-fucose by recombinant bacterial enzymes (Mattila *et al.*, 2000). Therefore, in order to generate GDP-D-rhamnose in abundance, exogenous GDP-D-mannose was added to the reaction mixture of the lysates of yeast strains expressing GMD and RMD. However, most of the exogenously added GDP-D-mannose was observed to convert into something other than GDP-D-rhamnose. It is likely that there are enzymes other than GMD in the crude yeast lysate, such as mannosyltransferases (Kojima *et al.*, 1999; Todorow *et al.*, 2000), which also compete for GDP-D-mannose. Furthermore, GDP-D-rhamnose might inhibit the activity of GMD via feedback inhibition similar to GDP-L-fucose (Bisso *et al.*, 1999; Somoza *et al.*, 2000; Sturla *et al.*, 1997). A recent study on recombinant *A. thaliana* GMD (named as MUR1) has demonstrated that GDP-D-rhamnose binds to *A. thaliana* GMD *in vitro*, although *A. thaliana* is not known to synthesize GDP-D-rhamnose (Mulichak *et al.*, 2002). Thus, one could speculate that the yeast lysate co-expressing GMD and RMD yielded a lower amount of GDP-D-rhamnose than the mixture of lysates of yeast strains expressing either GMD or RMD due to the feedback inhibition of GMD by GDP-D-rhamnose. Especially, since a similar phenomenon has also been seen when compared the GDP-L-fucose yields of corresponding lysates of yeast strains expressing *H. pylori* GMD and GMER (data not shown). However, this comparison is not unequivocal, because protein concentrations of the recombinant proteins were not determined. Purification of the GMD and RMD enzymes and optimization of reaction conditions might lead to more efficient conversion of GDP-D-mannose into GDP-D-rhamnose.

7.2.4. Conservation of RMD homologs among bacteria (II)

The amino acid sequence of RMD was used as a probe to find more putative RMD sequences from the nucleotide or protein databases based on the sequence similarity. Relatively high sequence similarities were found from the characterized *A. thermoaerophilus* RMD as well as from three other bacterial ORFs of *M. tuberculosis, Thiobacillus ferrooxidans* and *Xylella fastidiosa*. Of the three latter bacteria, *T. ferrooxidans* is the only one known to express D-rhamnose as a constituent of its LPS (Vinogradov *et al.*, 1994). Significant sequence similarities were also found with GMD, RmlB and GalE protein families. Therefore, selected gene sequences from the four enzyme families were aligned to evaluate the distance *inter se*. The mutation rate was used as a measure of distance between the sequences and standard phylogenetic tools were used to visualize the result. The analysis showed that while the different genes clustered according to their proposed function (Figure 6/II), their relatedness within the group was not much higher than between the groups. This was further analyzed by aligning the amino acid sequences from the RMD group: the sequences of *P. aeruginosa* and *A. thermoaerophilus* with proven RMD activities, and the ORFs of *T. ferrooxidans*, *M. tuberculosis* and *X. fastidiosa* (Figure 7/II). The sequences did not have any major stretches of similarity, but rather short patterns, most of which are also found in the

other three enzyme families (data not shown). Hence, prediction of enzymatic function of the putative enzymes of these protein families on the basis of sequence comparison may not be unequivocal, unless the sequence contains a typical motif, such as amino acid sequence GILFNHES commonly found in GMDs (I; Kneidinger *et al*., 2001; Somoza *et al*., 2000). Furthermore, before a reasonable comparison of evolutionary relationships can be made more structural or enzyme activity data for these protein families are required.

7.3. Methodological development for the analysis of nucleotide sugars (III)

7.3.1. Separation of nucleotide sugars by ion-pair reversed-phase HPLC (III)

An ion-pair reversed-phase chromatography with the volatile triethylammoniumacetate (TEAA) buffer, which is a well-established technique used for separation of nucleotides (Eriksson *et al*., 1986), was developed for the analysis of nucleotide sugars. The potential of reversed-phase HPLC with TEAA buffer as an ion-pairing reagent was evaluated with ten pure nucleotide sugars, *e*.g. GDP-D-mannose, GDP-D-rhamnose and GDP-L-fucose (Figure 2/III). Negatively charged nucleotide sugars bound the ion-pairing reagent and were retained on the HPLC column. They were eluted with a gradient of increasing acetonitrile concentration. Of the ten nucleotide sugars tested, eight were easily resolved, but UDP-*N*-acetyl-D-galactosamine and UDP-*N*-acetyl-D-glucosamine had the same retention time. In separation of deoxyhexose nucleotides, the method worked very well. Both GDP- and dTDP-deoxyhexoses were resolved from the nucleotide hexoses that are their starting materials in the biosynthetic reactions.

7.3.2. Ion-pair solid-phase extraction of nucleotide sugars from cell lysates (III)

If nucleotide sugars from cell lysates are to be analyzed by reversed-phase HPLC, they first need to be purified. Solid-phase extraction (SPE) columns containing graphitized carbon were used for this step. Activated carbon in the form of charcoal has been used as a preparative purification method for nucleotide sugars for decades (Okazaki *et al*., 1962). SPE columns containing graphitized carbon have been used for purification of oligosaccharides from salts, detergents, and proteins (Packer *et al*., 1998). In this study, nucleotide sugars were found to adsorb tightly to carbon SPE columns. They could be recovered quantitatively by adding the ion-pairing reagent TEAA to the organic elution solvent.

The recovery of nucleotide sugars in purification with carbon SPE columns was tested with pure nucleotide sugars. Nucleotide sugars were quantitatively adsorbed to carbon SPE columns. Salts and unwanted, weakly bound materials were washed off from columns with water and then with 25% acetonitrile and finally with 50 mM TEAA buffer. As mentioned above, both the ion-pair reagent TEAA and the organic solvent 25% acetonitrile were required for the elution of nucleotide sugars. TEAA was removed from the eluates by evaporation under vacuum prior to HPLC analysis. The recovery of pure nucleotide sugars was almost complete, being in the range of 93-100% (Table

1/III). The capacity of carbon columns was also tested with internal standard by adding known concentrations of GDP-L-fucose to *S. cerevisiae* YPH501 lysate. The recovery of GDP-L-fucose from the yeast lysate was almost 100% (Table 2/III), as with pure nucleotide sugars. Defined by protein concentration, about 1 mg of yeast cell lysate could be loaded into one 250 mg column without remarkable loss in recovery.

7.3.3. HPLC analysis of nucleotide sugars purified from lysates (I, II, III)

The newly developed reversed-phase HPLC in conjunction with the carbon SPE method were used to analyze the nucleotide sugars from the lysates of recombinant *S. cerevisiae* strains expressing bacterial enzymes synthesizing GDP-deoxyhexoses (I, II). Synthesized nucleotide sugars were identified by comparison of retention times to those of nucleotide sugar standards. Furthermore, some nucleotide sugar peaks obtained from yeast extracts were also collected and analyzed with MALDI-TOF MS (Table 3/III). In addition to endogenous UDP-*N*-acetylhexosamine of yeast, GDP-deoxyhexoses synthesized by heterologously expressed bacterial enzymes and some uncharacterized peaks were detected (Figure 3/III).

For most nucleotide sugars, the single-step purification of yeast lysates with carbon SPE columns prior to HPLC analysis was found to be enough. However, impurity peaks were seen to fuse with some nucleotide sugar peaks in the HPLC chromatograms (Figure 3/III). Most of these unknown peaks were removed with alkaline phosphatase treatment (Figure 4/III), which degrades nucleotides, but leaves nucleotide sugars intact. This suggests that the uncharacterized peaks seen after graphite SPE purification were mostly nucleotides. After the alkaline phosphatase treatment, samples were purified with DEAE anion exchange columns using volatile NH_4HCO_3.

Reversed-phase HPLC and carbon SPE method combined with alkaline phosphatase treatment and DEAE anion exchange purification were also used to analyze the enzyme activities of *P. aeruginosa* synthesizing GDP-deoxyhexoses. No significant GDP-sugar peaks were seen in the HPLC chromatogram of native *P. aeruginosa* (Figure 5/III, panel A). However, the addition of GDP-D-mannose to the *P. aeruginosa* lysate boosted the synthesis of GDP-D-rhamnose, which was detected in the HPLC chromatogram (Figure 5/III, panel B).

Methods for the analysis of nucleotide sugar profiles of different cells will be a useful tool in studying the regulation and abnormalities in glycosylation or in searching for new enzymes producing nucleotide sugars. The developed SPE and HPLC methods in sample processing and nucleotide sugar separation enable good separation of structurally similar sugar nucleotides, as seen in the analysis of lysates of recombinant *S. cerevisiae* strains and *P. aeruginosa*. These methods are suitable for both analytical and preparative separation of nucleotide sugars, and they allow automation and upscaling of sample volumes. Furthermore, SPE purification can be easier and more rapidly performed in comparison to other sample preparation methods of nucleotide sugars, such as deproteinization with boiling (Albermann *et al*., 2000), perchlorate (Martin *et al*., 1989), and organic solvents (Liljebjelke *et al*., 1995; Palmieri *et al*.,

1991; Rush and Waechter, 1995; Tomiya *et al*., 2001). Ion-pair SPE is a less frequently applied technique (Carson, 2000), but it offers many advantages over more conventional SPE. The use of volatile TEAA buffer eliminates the need for further desalting prior to HPLC, MS or NMR, which can be performed simply after evaporation of TEAA under vacuum. This is advantageous as there are a limited amount of nucleotide sugar standards commercially available, and MS or NMR may be needed to determine the precise structure of the nucleotide sugar of interest. Interestingly, it seems that the ion-pairing reagent TEAA works in opposite ways in carbon SPE and in reversed-phase HPLC by either decreasing or increasing retention, respectively. Most probably the developed SPE and HPLC could also be used in analysis and comparison of nucleotide profiles of different cell types.

7.4. *A. actinobacillus* GDP-6-deoxy-D-talose synthetase (GTS) (IV)

7.4.1. Sequence comparison of GMER, RMD and GTS (I, II, IV)

From the gene cluster associated with the serotype a-SPA of *A. actinomycetemcomitans* (Suzuki *et al*., 2000), a GDP-6-deoxy-D-talose synthetase *(gts*) gene was identified on the basis of its amino acid sequence similarity with closely related bacterial reductases responsible for the synthesis of other GDP-deoxyhexoses. The corresponding gene product contained an N-terminal coenzyme-binding pattern ThrGlyXXGlyXXGly, and an active-site pattern Ser-TyrXXXLys (Figure 2/IV), which are the most typical motifs of the SDR protein family (Jörnvall *et al*., 1995). The other conserved features of the SDR protein family, such as an NNAG motif or Asn111, were not found (Filling *et al*., 2002; Oppermann *et al*., 2001). Additionally, these former motifs of SDR protein family were present, while latter motifs were absent from the sequences of other bacterial enzymes using GDP-4-keto-6-deoxy-D-mannose as a substrate. Compared to those enzymes (Figure 2/IV), the putative GTS protein shared 32% identity with *E. coli* GMER (Tonetti *et al*., 1998) and 28% identity with *H. pylori* GMER (I). It also exhibited 30% identity with *A. thermoaerophilus* (Kneidinger *et al*., 2001) and *P. aeruginosa* RMDs (II).

The amino acid sequence of GTS was more closely compared to that of *E. coli* GMER. The crystallographic studies have indicated that a catalytic mechanism of GMER is based on the concerted action of the evolutionarily conserved residues Ser107, Tyr136 and Lys140 (Rizzi *et al*., 1998; Rosano *et al*., 2000). These amino acid residues were also present in the GTS sequence (Ser103, Tyr128 and Lys132). The GMER model also indicated that the GDP-4-keto-6-deoxy-D-mannose substrate might be located in the pocket facing Ser107, Ser108 and Cys109 residues (Rizzi *et al*., 1998; Rosano *et al*., 2000). Neither Ser108 nor Cys109 were found in the GTS sequence. Moreover, the *E. coli* GMER residues His179, which is suggested to play a role in the epimerisation reaction (Rosano *et al*., 2000), and Arg187, which is considered to be part of the substrate-binding cleft (Rizzi *et al*., 1998; Rosano *et al*., 2000), were absent from the GTS sequence. In addition, the His179 residue was not found from the RMDs of *A.*

thermoaerophilus and *P. aeruginosa*, whereas it was found from *H. pylori* GMER (Figure 2/IV). The His179 residue of GMER enzyme was equivalent to the Phe residue in RMD enzymes and GTS (Figure 2/IV), which are acting only as reductases. The sequence alignment of GMERs, RMDs and GTS also showed that the GMER Arg187 equivalent residue was Gln178 in RMDs and Ala173 in GTS (Figure 2/IV). Amino acid residue variations between the GDP-4-keto-6-deoxy-D-mannose exploiting enzymes may be related to the different stereospecificity of the reduction reaction or the order of reaction cascade (in the case of GDP-L-fucose synthesis epimerisation occurs prior reduction). GMD is required to synthesize a GDP-4-keto-6-deoxy-D-mannose substrate for GTS. Therefore, a putative *gmd* gene was also identified from the serotype a-SPA gene cluster of *A. actinomycetemcomitan*s. The corresponding gene product shared 20% identity with characterized GMD proteins and it contains the amino acid sequence GILFNHES which is commonly found in GMD (I; Kneidinger *et al*., 2001; Somoza *et al*., 2000). The characterization of *A. actinomycetemcomitans* GMD was not performed in this study.

7.4.2. Functional expression of GTS (IV)

The 885 bp *gts* gene was amplified from the chromosomal DNA of *A. actinomycetemcomitans* serotype a and subcloned under the anhydrotetracycline inducible *tet* promoter of the pASK-IBA5 expression vector in-frame with the N-terminal Strep-tag II. In this study, IBA's *E. coli* expression system was used for overexpression of GTS (Table 1), because it enables the single-step affinity chromatography purification of expressed Strep tagged fusion proteins and because GTS was not expressed in the *S. cerevisiae* expression system. *H. pylori gmd* was also subcloned into the same expression vector in-frame with N-terminal Strep-tag II, because the characterized *H. pylori* GMD was needed to generate GDP-4-keto-6-deoxy-D-mannose substrate for GTS. The resulting plasmids were used for overexpression of the fusion proteins in *E. coli* BL21(DE3). The heterologous expression of purified GMD and GTS was studied by SDS-PAGE. The molecular masses determined by SDS-PAGE for the purified, denaturated GMD and GTS corresponded to the calculated molecular masses of *ca.* 44 and 34 kDa, respectively (Figure 3/IV, lanes 3 and 4). No corresponding bands were detected from the vector control (Figure 3/IV, lanes 2). As judged from the negative control experiments with the vector control, the few contaminating proteins purified along with GMD and GTS were not responsible for the observed enzyme activity (Figure 4/IV).

7.4.3. Enzymatic activity of recombinant GTS (IV)

Incubation of recombinant *E. coli* lysates expressing GMD and GTS with exogenously added GDP-D-mannose resulted in a new peak in the HPLC profile (data not shown). This new compound was pooled from several HPLC runs and subjected to further analysis with MALDI-TOF MS and NMR. MALDI-TOF MS showed that the new molecule gave a single peak at *m/z* 588.08, which is identical to the mass of GDP-deoxyhexoses (calculated *m/z* for $[M-H]^-$ is 588.08).

The structure of the novel nucleotide sugar purified by HPLC was established to be GDP-6-deoxy-talose by ^{1}H NMR spectroscopy. Assignments of the proton signals (Figure 5/IV) were obtained from a DQFCOSY spectrum (Figure 6/IV, panel B) and proton-proton coupling constants $^{3}J_{H,H+1}$ (Table I/IV) were determined by a first order analysis. The ^{1}H chemical shifts of the hexosyl unit (Table I/IV) were clearly different from those obtained for GDP-D-rhamnose (Table 2/II) or GDP-L-fucose (Adelhorst and Whitesides, 1993). The small coupling constants between the ring protons of the hexosyl unit were characteristic of a mannoconfiguration at position C2 and of a galactoconfiguration at position C4 and thus confirms the hexosyl unit as 6-deoxy-talose. In addition, a cross-peak was observed in the DQFCOSY spectrum between H2 and H4 of the deoxytalosyl unit (Figure 6/IV, panel B). This four-bond coupling arises from the planar W orientation of the bonds (Figure 6/IV, panel A). Such long-range couplings are rarely observed in carbohydrates, because a similar pair of equatorial protons does not exist in the most common monosaccharide units. Therefore, the observed coupling further confirms the mutual orientation of H2 and H4 as equatorial-equatorial.

After having GDP-6-deoxy-D-talose standard characterized by MALDI-TOF MS and NMR (Figure 4/IV, lane E), nucleotide sugars formed in the reactions of purified Strep tagged GMD and GTS with exogenously added GDP-D-mannose and $NADP^{+}$ and/or NADPH were analyzed with reversed-phase HPLC (Figure 4/IV). In the HPLC analysis, the reaction mixture containing GMD gave a peak representing the GDP-4-keto-6-deoxy-D-mannose intermediate (Figure 4/IV, lane C). The presence of GDP-4-keto-6-deoxy-D-mannose in the reaction mixture was studied by a chemical reduction with $NaBH_4$. After the chemical reduction, two new peaks, GDP-D-rhamnose and GDP-6-deoxy-D-talose were detected with HPLC (data not shown). The GDP-4-keto-6-deoxy-D-mannose peak was not detected from the reaction mixture containing GMD and GTS, instead a novel peak with the same retention time as the GDP-6-deoxy-D-talose standard was observed (Figure 4/IV, lane D). GDP-4-keto-6-deoxy-D-mannose or GDP-6-deoxy-D-talose peaks were not detected from the vector control (Figure 4/IV, lane B). The data from the HPLC chromatograms was very solid, because carbon SPE purification of reaction mixtures containing purified enzymes together with cofactors and substrates yielded only GDP-sugars that indicated the biochemical activities of GMD and GTS (Figure 4/IV). The addition of NADPH as a cofactor was required for the GTS activity. Because no reverse reaction or other reaction products were detected in HPLC analysis, the conversion of GDP-4-keto-6-deoxy-D-mannose to GDP-6-deoxy-D-talose by GTS proceeded quantitatively and specifically. The identification and biochemical activity of *A. actinomycetemcomitans* GTS was also confirmed by an independent study by Suzuki and colleagues (2002).

A. thermoaerophilus GMD has been demonstrated to be a bifunctional enzyme acting as a GDP-D-mannose 4,6-dehydratase and GDP-4-keto-6-deoxy-D-mannose reductase (Kneidinger *et al*., 2001). In this study, the bifunctionality of purified *H. pylori* GMD was not observed suggesting that the specificity of GMD enzymes varies among bacterial species. Furthermore, the expressed GMD and GDP-4-keto-6-deoxy-D-

mannose utilizing enzymes were stable and active as fusion proteins with N-terminal c-myc-, FLAG- or Strep-tags. There are reports that the recombinant *E. coli* GMD, *V. cholerae* GMD and *E. coli* GMER have not been active or stable when expressed natively or as an N-terminal fusion with His-tag (Albermann *et al*., 2000; Albermann and Piepersberg, 2001; Sturla *et al*., 1997).

7.4.4. Synthesis of GDP-6-deoxy-D-talose by purified GTS (IV)

The yield of synthesized GDP-6-deoxy-D-talose by purified GMD and GTS after the purification steps was determined with HPLC. As calculated from the GMD reaction product, GDP-4-keto-6-deoxy-D-mannose, 79% of the substrate was converted into GDP-6-deoxy-D-talose. The remaining (21%) GDP-4-keto-6-deoxy-D-mannose was not detected in HPLC analysis probably due to the labile nature of GDP-4-keto-6-deoxy-D-mannose (Bonin and Reiter, 2000; Kneidinger *et al*., 2001). As calculated from GDP-D-mannose added to the reaction mixture containing GMD, 14% of GDP-D-mannose was converted into GDP-6-deoxy-D-talose by GTS. Optimization of reaction conditions might lead to a more efficient conversion of GDP-D-mannose into GDP-6-deoxy-D-talose. In general, purification of enzymes enables kinetic studies of enzyme reaction, but in this study the kinetic parameters of GTS were not analyzed due to the instability of GDP-4-keto-6-deoxy-D-mannose.

8. CONCLUSIONS AND FUTURE PROSPECTS

This study involved the molecular identification and enzymatic characterization of nucleotide sugar synthesizing enzymes, that produce GDP-L-fucose, GDP-D-rhamnose and GDP-6-deoxy-D-talose. Enzymes were identified from the pathogenic bacteria *H. pylori*, *P. aeruginosa* and *A. actinomycetemcomitans* serotype a, in which deoxyhexoses L-fucose, D-rhamnose, 6-deoxy-D-talose are constituents of LPS, respectively. The specific functions of LPS molecules containing these deoxyhexoses in the course of colonization and infection are unknown. However, the relevance of LPS in pathogenesis of these bacteria is under extensive studies.

The biosyntheses of GDP-L-fucose, GDP-D-rhamnose and GDP-6-deoxy-D-talose have received increased interest after L-fucose, D-rhamnose, and 6-deoxy-D-talose were shown to be substantial cell wall components of several human pathogens. Some of these pathogens, which can cause community-acquired diseases or nosocomial infections, are multiple drug-resistant. Although a large number of antibacterial drugs are available, most of them target the same cellular process or even the same target enzyme leading to the potential for cross-resistance. Multiple drug-resistance and cross-resistance of pathogens creates a high demand for therapeutics acting with new strategies. New drugs with novel functions may be discovered from the biosynthetic pathways of glycoconjugates or glycosyltransferases. If the roles of fucosylation/ rhamnosylation/talosylation are determined to be essential for the viability of the microorganism or the host – pathogen interaction, the enzymes involved in the biosynthesis of these glycoforms could be suitable targets for antibacterial agents. Humans lack rhamnosylation and talosylation and would thus most likely not suffer from the inhibition of metabolism related to these glycoforms.

L-fucose is the only representative of these three deoxyhexoses also found in eukaryotes. Nevertheless, the biosynthetic *de novo* pathway of GDP-L-fucose could also be considered as a potential therapeutic target, because humans, as well as other eukaryotes, have an alternative pathway for GDP-L-fucose biosynthesis. This pathway is a result of the 'salvage' metabolism, which uses fucose derived from lysosomal degradation or nutrition (reviewed in Bulter and Elling, 1999). Smith and colleagues (2002) have shown that the *de novo* pathway knock-out mice grew normally when fed with fucose-supplemented food. If in humans, dietary or salvaged fucose also makes a substantial contribution to the fucosylation, defect caused by the short-term inhibition of the enzymes involved in the *de novo* pathway of GDP-L-fucose, might not cause suffering in humans. The *de novo* biosynthesis of GDP-L-fucose can also be a target for therapeutical treatment of pathological conditions of abnormal selectin-mediated cell-to-cell interaction, such as leukocyte trafficking into sites of inflammation (Kirveskari *et al*., 2001; reviewed in Lowe, 2001) or in bacterial adherence to host fucosylated glycans (Herron *et al*., 2000; reviewed in Hooper and Gordon, 2001). Inhibition of the *de novo* biosynthesis of GDP-L-fucose may reduce the effective synthesis of fucosylated

ligands and thus interfere cell-to-cell interaction or cell adhesion in these pathological conditions.

Understanding the mechanism of biosynthesis of these nucleotide sugars should aid in the rational design of drugs with potential in antibacterial or anti-inflammatory chemotherapy. Hence, the detailed studies of the discrete catalytic mechanisms of the reactions and the specific regulation sites of GMD, GMER, RMD, and GTS are needed. Analysis of these closely related enzymes would also contribute to the understanding of catalytic mechanism of other dehydratases, epimerases or reductases belonging to the SDR protein family that could also be considered as targets for antimicrobial agents, such as the enzymes involved in the biosynthesis of dTDP-L-rhamnose. Disruption of the dTDP-L-rhamnose pathway has the attenuated virulence or influenced the viability of pathogenic bacteria, *e.g. V. cholerae*, *Enterococcus feacalis* and *Streptococcus mutans* (Chiang and Mekalanos, 1999; Xu *et al*., 2000; Yamashita *et al*., 1999). These observations elucidate the importance in bacterial pathogenicity of enzymes involved in the biosynthesis of LPS or capsular sugars.

The prevalence of the GDP-L-fucose, GDP-D-rhamnose, and GDP-6-deoxy-D-talose pathways is not high, but narrow-spectrum drugs may be of interest in certain disease states like chronic infections that require long-term treatment. Furthermore, narrow-spectrum agents would also minimize the spread of drug resistance to other pathogens. It is worth mentioning that in addition to the GDP-L-fucose, GDP-D-rhamnose, and GDP-6-deoxy-D-talose pathways, the inhibition of GMD activity would also influence the biosynthesis of GDP-D-perosamine and GDP-colitose. D-perosamine is a constituent of glycoconjugates in several Gram-positive and Gram-negative bacteria, *e.g. E. coli* O:157, *V. cholerae* O:1 and *Y. enterocolitica* O:9 (Bilge *et al*., 1996; Villeneuve *et al*., 2000; reviewed in Skurnik and Zhang, 1996). Colitose is a constituent of LPS in Gram-negative bacteria, *e.g. E. coli* O:111, *S. enterica* O:35 and *Y. pseudotuberculosis* O:6 and O:7 (Wang and Reeves, 2000; reviewed in Skurnik and Zhang, 1996). Humans lack metabolism for both of these nucleotide sugars.

Naturally, before the biosynthetic pathways of GDP-L-fucose, GDP-D-rhamnose, and GDP-6-deoxy-D-talose could be considered as therapeutic targets, the possible roles of fucosylation/rhamnosylation/talosylation in the viability or virulence of pathogenic bacteria should be determined. Pathogenesis models will permit the identification of the virulence factors as well as the elucidation of the efficacy of potential chemotherapeutic agent. For instance, there are several pathogenesis models for *H. pylori* (reviewed in Nedrud, 1999), *P. aeruginosa* (reviewed in Andes and Craig, 1998; Stotland *et al*., 2000) and *A. actinomycetemcomitans* (Kawai *et al*., 1998; Sosroseno and Herminajeng, 2002). The ability of *P. aeruginosa* to infect a variety of plant, invertebrate and vertebrate hosts has also enabled the development of rapid pathogenesis models using for example *A. thaliana* or *Caenorhabditis elegans* as a host (Rahme *et al*., 1995; Tan *et al*., 1999). Furthermore, it has been observed that several factors that affect the *P. aeruginosa* – plant/nematode infections also operate in the *P. aeruginosa* – mammal infections (Mahajan-Miklos *et al*., 1999; Rahme *et al*., 2000). Pathogenesis models and the developments in microarray technology and the whole-genome analysis

of host organisms will also allow the role of the host factors to be evaluated. This is especially important when studying the influence of inhibition of metabolic pathways which exist both in pathogen and host organisms, as is the case of the GDP-L-fucose pathway.

Research groups studying the relevance of glycoforms of various bacteria would benefit from the availability of building blocks required for the synthesis of glycosylated molecules. Before these molecules can be synthesized *in vitro*, the activated nucleotide sugars and the corresponding glycosyltransferases catalyzing the formation of specific glycosidic linkages are needed. Fucosyltransferases with different acceptor and linkage specificities have been characterized from a limited number of bacteria (reviewed in Oriol *et al.*, 1999; Wang *et al.*, 2000), however the enzymatic characterization of identified D-rhamnosyltrasferases (Rocchetta *et al.*, 1998) or identification and characterization of deoxytalosyltransferases remains to be done. This study provides three different nucleotide sugars, which can be utilized in characterization of glycosyltransferases or in oligosaccharide synthesis.

This study also provides a novel method for the analysis of the cellular nucleotide sugar pool. This newly developed reversed-phase HPLC can also be used in preparative oligosaccharide synthesis or to study regulation or inhibition of enzymes involved in the synthesis of nucleotide sugars. In general, there are not many methods available for carbohydrate chemistry in comparison to available methods for genomics or proteomics. Hence, any methodological development for studies of carbohydrate chemistry or "glycomics" is valuable.

ACKNOWLEDGEMENTS

This study was carried out in the Rational Drug Design –research program at the Biomedicum Helsinki and at the Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki. I wish to thank Professor Seppo Meri the present Head of the Department of Bacteriology and Immunology and Docent Risto Renkonen for providing me excellent working facilities.

I owe much gratitude to Risto Renkonen for introducing the world of glycobiology to me, for his interest in my work and for being an optimistic, determined and encouraging supervisor.

Docent Marko Salmi and Professor Mikael Skurnik are warmly thanked for critically reviewing this thesis.

Emeritus professor Ossi Renkonen is acknowleged for his interest in my work.

I also wish to thank all my co-authors: Nina Järvinen, Hannu Maaheimo, Pirkko Mattila, Leena Penttilä, Christophe Roos, Jarkko Räbinä, and Emma Savilahti for their valuable collaboration. I especially wish to thank Jarkko Räbinä for his constructive suggestions while planning the experiments and writing my articles and thesis, and Leena Penttilä for her much appreciated advices and discussions about and beyond science. I am most grateful to my dear friend Nina Järvinen with whom I have enjoyed and tried to solve many everyday challenges we faced in the lab. Nina, thank you!

Warm thanks are owned to the former and present members of the RR-team for collaboration, good companionship and joyful coffee breaks. Special thanks are due to “the catalytic triad” of Nina Järvinen, Jaana Niittymäki and Solveig Sjöblom for their friendship and support. Tuula Kallioinen and Kikka Kauranen are thanked for their valuabe help. Kikka is especially thanked for starting my experiments early in the morning while I have still been sleeping. I also want to express my thanks to Satu Bruun, Laura Huopaniemi, Juha Kirveskari, and Riika Salmela for their nice company and hilarious sense of humor that has cheered me up so often.

I wish to acknowledge Laura Mannonen, my virtual twin, for sharing my interest in microbiology and computer science. Laura is also thanked for her valuable help and support during this spring. The members of Pelastakaa Köyhät Maisterit, Mirko Brummer, Anssi Rantakari and Anne Tammiruusu ,are acknowledged for their support starting from the early days of my microbiology studies. Anne is also thanked for her refreshing, almost daily e-mails. Massimiliano Gentle is thanked for the many encouraging discussions we have had during this spring. My very warm thanks are due to my other friends outside the science community for their care and support.

My heartfelt thanks go to my parents, Merja and Rauno, and to my siblings and their families, Rami, Sari, Riku, Roni, Sanna, Matti, Hemppa, Samppa, Onsku, Jonna, Risto,

and Riina, for their love and encouragement. Jonna is also thanked for preparing the nice layout of this thesis. My sincerest gratitude belongs to my dear Jukka, who has supported my both careers and patiently understod my long working days. Jukka, thank you!

This work was financially support by the Research and Science Foundation of Farmos, the Paulo Foundation, Academy of Finland, Technology Development Center (TEKES), and the Sigrid Juselius Foundation.

Kiitos kaikille!

Helsinki, May 2003

Minna Mäki

REFERENCES

Abe, M., Hashimoto, H. & Yoda, K. (1999). Molecular characterization of Vig4/Vrg4 GDP-mannose transporter of the yeast *Saccharomyces cerevisiae*. *FEBS Letters.* **458**, 309-12.

Adelhorst, K. & Whitesides, G. M. (1993). Large-scale synthesis of β-L-fucopyranosyl phosphate and the preparation of GDP-β-L-fucose. *Carbohydrate Research.* **242**, 69-76.

Albermann, C., Distler, J. & Piepersberg, W. (2000). Preparative synthesis of GDP-beta-L-fucose by recombinant enzymes from enterobacterial sources. *Glycobiology.* **10**, 875-81.

Albermann, C. & Piepersberg, W. (2001). Expression and identification of the RfbE protein from *Vibrio cholerae* O1 and its use for the enzymatic synthesis of GDP-D-perosamine. *Glycobiology.* **11**, 655-61.

Allard, S. T., Beis, K., Giraud, M. F., Hegeman, A. D., Gross, J. W., Wilmouth, R. C., Whitfield, C., Graninger, M., Messner, P., Allen, A. G., Maskell, D. J. & Naismith, J. H. (2002). Toward a structural understanding of the dehydratase mechanism. *Structure.* **10**, 81-92.

Allard, S. T., Giraud, M. F. & Naismith, J. H. (2001). Epimerases: structure, function and mechanism. *Cellular & Molecular Life Sciences.* **58**, 1650-65.

Allard, S. T., Giraud, M. F., Whitfield, C., Graninger, M., Messner, P. & Naismith, J. H. (2001). The crystal structure of dTDP-D-Glucose 4,6-dehydratase (RmlB) from *Salmonella enterica* serovar Typhimurium, the second enzyme in the dTDP-L-rhamnose pathway. *Journal of Molecular Biology.* **307**, 283-95.

Allard, S. T., Giraud, M. F., Whitfield, C., Messner, P. & Naismith, J. H. (2000). The purification, crystallization and structural elucidation of dTDP-D-glucose 4,6-dehydratase (RmlB), the second enzyme of the dTDP-L-rhamnose synthesis pathway from *Salmonella enterica* serovar typhimurium. *Acta Crystallographica Section D-Biological Crystallography.* **56**, 222-5.

Alm, R. A., Ling, L. S., Moir, D. T., King, B. L., Brown, E. D., Doig, P. C., Smith, D. R., Noonan, B., Guild, B. C., deJonge, B. L., Carmel, G., Tummino, P. J., Caruso, A., Uria-Nickelsen, M., Mills, D. M., Ives, C., Gibson, R., Merberg, D., Mills, S. D., Jiang, Q., Taylor, D. E., Vovis, G. F. & Trust, T. J. (1999). Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. *Nature.* **397**, 176-80.

Amano, K., Nishihara, T., Shibuya, N., Noguchi, T. & Koga, T. (1989). Immunochemical and structural characterization of a serotype-specific polysaccharide antigen from *Actinobacillus actinomycetemcomitans* Y4 (serotype b). *Infection & Immunity.* **57**, 2942-6.

Andes, D. R. & Craig, W. A. (1998). Pharmacodynamics of fluoroquinolones in experimental models of endocarditis. *Clinical Infectious Diseases.* **27**, 47-50.

Andrianopoulos, K., Wang, L. & Reeves, P. R. (1998). Identification of the fucose synthetase gene in the colanic acid gene cluster of *Escherichia coli* K-12. *Journal of Bacteriology* **180**, 998-1001.

Appelmelk, B. J., Martin, S. L., Monteiro, M. A., Clayton, C. A., McColm, A. A., Zheng, P., Verboom, T., Maaskant, J. J., van den Eijnden, D. H., Hokke, C. H., Perry, M. B., Vandenbroucke-Grauls, C. M. & Kusters, J. G. (1999). Phase variation in *Helicobacter pylori* lipopolysaccharide due to changes in the lengths of poly(C) tracts in alpha3-fucosyltransferase genes. *Infection & Immunity.* **67**, 5361-6.

Appelmelk, B. J., Monteiro, M. A., Martin, S. L., Moran, A. P. & Vandenbroucke-Grauls, C. M. (2000). Why *Helicobacter pylori* has Lewis antigens. *Trends in Microbiology.* **8**, 565-70.

Appelmelk, B. J. & Vandenbroucke-Grauls, C. M. (2000). *H. pylori* and Lewis antigens. *Gut.* **47**, 10-1.

Arsenault, T. L., Huges, D. W., MacLean, D. B., Szarek, W. A., Kropinski, A. M. & Lam, J. S. (1991). Structural studies on the polysaccharide portion of 'A-band' lipopolysaccharide from a mutant (AK14O1) of *Pseudomonas aeruginosa* strain PAO1. *Canadian Journal of Chemistry.* **69**, 1273-1280.

Aspinall, G. O., Monteiro, M. A., Pang, H., Walsh, E. J. & Moran, A. P. (1996). Lipopolysaccharide of the *Helicobacter pylori* type strain NCTC 11637 (ATCC 43504): structure of the O antigen chain and core oligosaccharide regions. *Biochemistry.* **35**, 2489-97.

Barua, S., Yamashino, T., Hasegawa, T., Yokoyama, K., Torii, K. & Ohta, M. (2002). Involvement of surface polysaccharides in the organic acid resistance of Shiga Toxin-producing *Escherichia coli* O157:H7. *Molecular Microbiology.* **43**, 629-40.

Becker, D. J. & Lowe, J. B. (1999). Leukocyte adhesion deficiency type II. *Biochimica et Biophysica Acta.* **1455**, 193-204.

Berg, D. E., Hoffman, P. S., Appelmelk, B. J. & Kusters, J. G. (1997). The *Helicobacter pylori* genome sequence: genetic factors for long life in the gastric mucosa. *Trends in Microbiology.* **5**, 468-74.

Bilge, S. S., Vary, J. C., Jr., Dowell, S. F. & Tarr, P. I. (1996). Role of the *Escherichia coli* O157:H7 O side chain in adherence and analysis of an *rfb* locus. *Infection & Immunity.* **64**, 4795-801.

Bisso, A., Sturla, L., Zanardi, D., De Flora, A. & Tonetti, M. (1999). Structural and enzymatic characterization of human recombinant GDP-D-mannose-4,6-dehydratase. *FEBS Letters.* **456**, 370-4.

Blankenfeldt, W., Asuncion, M., Lam, J. S. & Naismith, J. H. (2000). The structural basis of the catalytic mechanism and regulation of glucose-L-phosphate thymidylyltransferase (RmlA). *EMBO Journal.* **19**, 6652-63.

Blankenfeldt, W., Kerr, I. D., Giraud, M. F., McMiken, H. J., Leonard, G., Whitfield, C., Messner, P., Graninger, M. & Naismith, J. H. (2002). Variation on a theme of SDR. dTDP-6-deoxy-L-*lyxo*-4-hexulose reductase (RmlD) shows a new Mg2+-dependent dimerization mode. *Structure* **10**, 773-86.

Bonin, C. P., Potter, I., Vanzin, G. F. & Reiter, W. D. (1997). The MUR1 gene of *Arabidopsis thaliana* encodes an isoform of GDP-D-mannose-4,6-dehydratase, catalyzing the first step in the *de novo* synthesis of GDP-L-fucose. *Proceedings of the National Academy of Sciences of the United States of America.* **94**, 2085-90.

Bonin, C. P. & Reiter, W. D. (2000). A bifunctional epimerase-reductase acts downstream of the MUR1 gene product and completes the de novo synthesis of GDP-L-fucose in *Arabidopsis. Plant Journal.* **21**, 445-54.

Bonin, C. P. & Reiter, W. D. (2000). A bifunctional epimerase-reductase acts downstream of the MUR1 gene product and completes the *de novo* synthesis of GDP-L-fucose in *Arabidopsis*. *Plant Journal.* **21**, 445-454.

Breton, C., Oriol, R. & Imberty, A. (1998). Conserved structural features in eukaryotic and prokaryotic fucosyltransferases. *Glycobiology.* **8**, 87-94.

Broschat, K. O., Chang, S. & Serif, G. (1985). Purification and characterization of GDP-D-mannose 4,6-dehydratase from porcine thyroid. *European Journal of Biochemistry.* **153**, 397-401.

Bulet, P., Hoflack, B., Porchet, M. & Verbert, A. (1984). Study of the conversion of GDP-mannose into GDP-fucose in Nereids: a biochemical marker of oocyte maturation. *European Journal of Biochemistry.* **144**, 255-9.

Bulter, T. & Elling, L. (1999). Enzymatic synthesis of nucleotide sugars. *Glycoconjugate Journal.* **16**, 147-59.

Carlson, R. W., Price, N. P. & Stacey, G. (1994). The biosynthesis of rhizobial lipo-oligosaccharide nodulation signal molecules. *Molecular Plant-Microbe Interactions.* **7**, 684-95.

Carson, M. C. (2000). Ion-pair solid-phase extraction. *Journal of Chromatography. A.*. **885**, 343-50.

Cerantola, S. & Montrozier, H. (1997). Structural elucidation of two polysaccharides present in the lipopolysaccharide of a clinical isolate of *Burkholderia cepacia*. *European Journal of Biochemistry.* **246**, 360-6.

Chan, N. W., Stangier, K., Sherburne, R., Taylor, D. E., Zhang, Y., Dovichi, N. J. & Palcic, M. M. (1995). The biosynthesis of Lewis X in *Helicobacter pylori*. *Glycobiology.* **5**, 683-8.

Chang, S., Duerr, B. & Serif, G. (1988). An epimerase-reductase in L-fucose synthesis. *Journal of Biological Chemistry.* **263**, 1693-7.

Chiang, S. L. & Mekalanos, J. J. (1999). rfb mutations in *Vibrio cholerae* do not affect surface production of toxin-coregulated pili but still inhibit intestinal colonization. *Infection & Immunity.* **67**, 976-80.

Christendat, D., Saridakis, V., Dharamsi, A., Bochkarev, A., Pai, E. F., Arrowsmith, C. H. & Edwards, A. M. (2000). Crystal structure of dTDP-4-keto-6-deoxy-D-hexulose 3,5-epimerase from *Methanobacterium thermoautotrophicum* complexed with dTDP. *Journal of Biological Chemistry.* **275**, 24608-12.

Christian, R., Schulz, G., Unger, F. M., Messner, P., Kupcu, Z. & Sleytr, U. B. (1986). Structure of a rhamnan from the surface-layer glycoprotein of *Bacillus stearothermophilus* strain NRS 2004/3a. *Carbohydrate Research.* **150**, 265-72.

de Vries, T., Knegtel, R. M., Holmes, E. H. & Macher, B. A. (2001). Fucosyltransferases: structure/function studies. *Glycobiology.* **11**, 119R-128R.

Edwards, N. J., Monteiro, M. A., Faller, G., Walsh, E. J., Moran, A. P., Roberts, I. S. & High, N. J. (2000). Lewis X structures in the O antigen side-chain promote adhesion of *Helicobacter pylori* to the gastric epithelium. *Molecular Microbiology.* **35**, 1530-9.

Engels, W., Endert, J., Kamps, M. A. & van Boven, C. P. (1985). Role of lipopolysaccharide in opsonization and phagocytosis of *Pseudomonas aeruginosa*. *Infection & Immunity.* **49**, 182-9.

Eriksson, S., Glad, G., Pernemalm, P. A. & Westman, E. (1986). Separation of DNA restriction fragments by ion-pair chromatography. *Journal of Chromatography. A.*. **359**, 265-74.

Etzioni, A., Sturla, L., Antonellis, A., Green, E. D., Gershoni-Baruch, R., Berninsone, P. M., Hirschberg, C. B. & Tonetti, M. (2002). Leukocyte adhesion deficiency (LAD) type II/carbohydrate deficient glycoprotein (CDG) IIc founder effect and genotype/phenotype correlation. *American Journal of Medical Genetics.* **110**, 131-5.

Filling, C., Berndt, K. D., Benach, J., Knapp, S., Prozorovski, T., Nordling, E., Ladenstein, R., Jörnvall, H. & Oppermann, U. (2002). Critical Residues for Structure and Catalysis in Short-chain Dehydrogenases/Reductases. *Journal of Biological Chemistry.* **277**, 25677-25684.

Fives-Taylor, P. M., Meyer, D. H., Mintz, K. P. & Brissette, C. (2000). Virulence factors of *Actinobacillus actinomycetemcomitans*. *Periodontology.* **20**, 136-67.

Ge, Z., Chan, N. W., Palcic, M. M. & Taylor, D. E. (1997). Cloning and heterologous expression of an alpha1,3-fucosyltransferase gene from the gastric pathogen *Helicobacter pylori*. *Journal of Biological Chemistry.* **272**, 21357-63.

Gerhard, M., Rad, R., Prinz, C. & Naumann, M. (2002). Pathogenesis of *Helicobacter pylori* infection. *Helicobacter.* **7**, 17-23.

Ginsburg, V. (1960). Formation of guanosine diphosphate L-fucose from guanosine diphosphate D-mannose. *Journal of Biological Chemistry.* **253**, 2196-2201.

Giraud, M. F., Leonard, G. A., Field, R. A., Berlind, C. & Naismith, J. H. (2000). RmlC, the third enzyme of dTDP-L-rhamnose pathway, is a new class of epimerase. *Nature Structural Biology.* **7**, 398-402.

Giraud, M. F., McMiken, H. J., Leonard, G. A., Messner, P., Whitfield, C. & Naismith, J. H. (1999). Overexpression, purification, crystallization and preliminary structural study of dTDP-6-deoxy-L-*lyxo*-

4-hexulose reductase (RmlD), the fourth enzyme of the dTDP-L-rhamnose synthesis pathway, from *Salmonella enterica* serovar Typhimurium. *Acta Crystallographica Section D-Biological Crystallography.* **55**, 2043-6.

Giraud, M.-F. & Naismith, J. H. (2000). The rhamnose pathway. *Current Opinion in Structural Biolology.* **10**, 687-696.

Gmur, R., McNabb, H., van Steenbergen, T. J., Baehni, P., Mombelli, A., van Winkelhoff, A. J. & Guggenheim, B. (1993). Seroclassification of hitherto nontypeable *Actinobacillus actinomycetemcomitans* strains: evidence for a new serotype e. *Oral Microbiology and Immunology.* **8**, 116-20.

Graninger, M., Nidetzky, B., Heinrichs, D. E., Whitfield, C. & Messner, P. (1999). Characterization of dTDP-4-dehydrorhamnose 3,5-epimerase and dTDP-4-dehydrorhamnose reductase, required for dTDP-L-rhamnose biosynthesis in *Salmonella enterica* serovar Typhimurium LT2. *Journal of Biological Chemistry.* **274**, 25069-77.

Hashimoto, H., Sakakibara, A.Yamasaki, M. & Yoda, K. (1997) *Saccharomyces cerevisiae* VIG9 encodes GDP-mannose pyrophosphorylase, which is essential for protein glycosylation. *Journal of Biological Chemistry*. **272**, 16308-16314.

Henderson, B., Wilson, M., Sharp, L. & Ward, J. M. (2002). *Actinobacillus actinomycetemcomitans*. *Journal of Medical Microbiology.* **51**, 1013-20.

Herron, M. J., Nelson, C. M., Larson, J., Snapp, K. R., Kansas, G. S. & Goodman, J. L. (2000). Intracellular parasitism by the human granulocytic ehrlichiosis bacterium through the P-selectin ligand, PSGL-1. *Science.* **288**, 1653-6.

Hoffman, J., Lindberg, B. & Brubaker, R. R. (1980). Structural studies of the O-specific side-chains of the lipopolysaccharide from *Yersinia enterocolitica* Ye 128. *Carbohydrate Research.* **78**, 212-4.

Hooper, L. V. & Gordon, J. I. (2001). Glycans as legislators of host-microbial interactions: spanning the spectrum from symbiosis to pathogenicity. *Glycobiology.* **11**, 1R-10R.

Humphries, H. E. & High, N. J. (2002). The role of licA phase variation in the pathogenesis of invasive disease by *Haemophilus influenzae* type b. *FEMS Immunology & Medical Microbiology.* **34**, 221-30.

Jin, Q., Yuan, Z., Xu, J., Wang, Y., Shen, Y., Lu, W., Wang, J., Liu, H., Yang, J., Yang, F., Zhang, X., Zhang, J., Yang, G., Wu, H., Qu, D., Dong, J., Sun, L., Xue, Y., Zhao, A., Gao, Y., Zhu, J., Kan, B., Ding, K., Chen, S., Cheng, H., Yao, Z., He, B., Chen, R., Ma, D., Qiang, B., Wen, Y., Hou, Y. & Yu, J. (2002). Genome sequence of *Shigella flexneri* 2a: insights into pathogenicity through comparison with genomes of *Escherichia coli* K12 and O157. *Nucleic Acids Research.* **30**, 4432-41.

Johnson, D. A. & Liu, H. (1998). Mechanisms and pathways from recent deoxysugar biosynthesis research. *Current Opinion in Chemical Biology.* **2**, 642-9.

Jörnvall, H., Persson, B., Krook, M., Atrian, S., Gonzalez-Duarte, R., Jeffery, J. & Ghosh, D. (1995). Short-chain dehydrogenases/reductases (SDR). *Biochemistry.* **34**, 6003-13.

Kaplan, J. B., Perry, M. B., MacLean, L. L., Furgang, D., Wilson, M. E. & Fine, D. H. (2001). Structural and genetic analyses of O polysaccharide from *Actinobacillus actinomycetemcomitans* serotype f. *Infection and Immunity.* **69**, 5375-84.

Kawai, T., Shimauchi, H., Eastcott, J. W., Smith, D. J. & Taubman, M. A. (1998). Antigen direction of specific T-cell clones into gingival tissues. *Immunology.* **93**, 11-9.

Kido, N., Torgov, V. I., Sugiyama, T., Uchiya, K., Sugihara, H., Komatsu, T., Kato, N. & Jann, K. (1995). Expression of the O9 polysaccharide of *Escherichia coli*: sequencing of the *E. coli* O9 *rfb* gene cluster, characterization of mannosyl transferases, and evidence for an ATP-binding cassette transport system. *Journal of Bacteriology.* **177**, 2178-87.

Kirveskari, J., Paavonen, T., Häyry, P. & Renkonen, R. (2000). *De novo* induction of endothelial L-selectin ligands during kidney allograft rejection. *Journal of the American Society of Nephrology.* **11**, 2358-65.

Kirveskari, J., Vesaluoma, M. H., Moilanen, J. A. O., Tervo, T. M. T., Petroll, M. W., Linnolahti, E. & Renkonen, R. (2001). A novel non-invasive, *in vivo* technique for the quantification of leukocyte rolling and extravasation at sites of inflammation in human patients. *Nature Medicine.* **7**, 376-379.

Kiska, D. L. & Gilligan, P. H. (1999). *Pseudomonas.* In *Manual of Clinical Microbiology*, pp. 517-525. Edited by P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover & R. H. Yolken. Washington, D.C.: American Society for Microbiology Press.

Kneidinger, B., Graninger, M., Adam, G., Puchberger, M., Kosma, P., Zayni, S. & Messner, P. (2001). Identification of two GDP-6-deoxy-D-*lyxo*-4-hexulose reductases synthesizing GDP-D-rhamnose in *Aneurinibacillus thermoaerophilus* L420-91^{T}*. *Journal of Biological Chemistry.* **276**, 5577-5583.

Knirel, Y. A. & Kochetkov, N. K. (1994). The structure of lipopolysaccharides of gram-negative bacteria. III. The structure of O-antigens: a review. *Biochemistry.* **59**, 1325-1382.

Kocharova, N. A., Knirel, Y. A., Widmalm, G., Jansson, P. E. & Moran, A. P. (2000). Structure of an atypical O-antigen polysaccharide of *Helicobacter pylori* containing a novel monosaccharide 3-C-methyl-D-mannose. *Biochemistry.* **39**, 4755-60.

Kojima, H., Hashimoto, H. & Yoda, K. (1999). Interaction among the subunits of Golgi membrane mannosyltransferase complexes of the yeast *Saccharomyces cerevisiae*. *Bioscience Biotehnology and Biochemistry.* **63**, 1970-1976.

Lam, M. Y., McGroarty, E. J., Kropinski, A. M., MacDonald, L. A., Pedersen, S. S., Hoiby, N. & Lam, J. S. (1989). Occurrence of a common lipopolysaccharide antigen in standard and clinical strains of *Pseudomonas aeruginosa*. *Journal of Clinical Microbiology.* **27**, 962-7.

Lamrabet, Y., Bellogin, R. A., Cubo, T., Espuny, R., Gil, A., Krishnan, H. B., Megias, M., Ollero, F. J., Pueppke, S. G., Ruiz-Sainz, J. E., Spaink, H. P., Tejero-Mateo, P., Thomas-Oates, J. & Vinardell, J. M. (1999). Mutation in GDP-fucose synthesis genes of *Sinorhizobium fredii* alters Nod factors and significantly decreases competitiveness to nodulate soybeans. *Molecular Plant-Microbe Interactions.* **12**, 207-17.

Lerouge, I. & Vanderleyden, J. (2002). O-antigen structural variation: mechanisms and possible roles in animal/plant-microbe interactions. *FEMS Microbiology Reviews.* **26**, 17-47.

Liao, T. H. & Barber, G. A. (1972). Purification of guanosine 5'-diphosphate D-mannose oxidoreductase from *Phaseolus vulgaris*. *Biochimica et Biophysica Acta.* **276**, 85-93.

Liljebjelke, K., Adolphson, R., Baker, K., Doong, R. L. & Mohnen, D. (1995). Enzymatic synthesis and purification of uridine diphosphate [14C]galacturonic acid: a substrate for pectin biosynthesis. *Analytical Biochemistry.* **225**, 296-304.

LiPuma, J. J. (2000). Expanding microbiology of pulmonary infection in cystic fibrosis. *Pediatric Infectious Disease Journal.* **19**, 473-474.

Lowe, J. B. (1997). Selectin ligands, leukocyte trafficking, and fucosyltransferase genes. *Kidney International.* **51**, 1418-26.

Lowe, J. B. (2001). Glycosylation, immunity, and autoimmunity. *Cell.* **104**, 809-12.

Luhn, K., Wild, M. K., Eckhardt, M., Gerardy-Schahn, R. & Vestweber, D. (2001). The gene defective in leukocyte adhesion deficiency II encodes a putative GDP-fucose transporter. *Nature Genetics.* **28**, 69-72.

Lyczak, J. B., Cannon, C. L. & Pier, G. B. (2002). Lung infections associated with cystic fibrosis. *Clinical Microbiology Reviews.* **15**, 194-222.

Mahajan-Miklos, S., Tan, M. W., Rahme, L. G. & Ausubel, F. M. (1999). Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa-Caenorhabditis elegans* pathogenesis model. *Cell.* **96**, 47-56.

Mahenthiralingam, E., Baldwin, A. & Vandamme, P. (2002). *Burkholderia cepacia* complex infection in patients with cystic fibrosis. *Journal of Medical Microbiology.* **51**, 533-8.

Maier, R. M. & Soberon-Chavez, G. (2000). *Pseudomonas aeruginosa* rhamnolipids: biosynthesis and potential applications. *Applied Microbiology & Biotechnology.* **54**, 625-33.

Markovitz, A. (1964). Biosynthesis of guanosine diphospate D-rhamnose and guanosine diphosphate D-talomethylose from guanosine diphosphate alpha-D-mannose. *Journal of Biological Chemistry.* **239**, 2091-2098.

Marshall, B. (2002). *Helicobacter pylori*: 20 years on. *Clinical Medicine.* **2**, 147-52.

Martin, A., Ruggiero-Lopez, D., Broquet, P., Richard, M. & Louisot, P. (1989). High-performance liquid chromatographic study of GDP-mannose and GDP-fucose metabolism. *Journal of Chromatography. A.*. **497**, 319-25.

Martin, S. L., Edbrooke, M. R., Hodgman, T. C., van den Eijnden, D. H. & Bird, M. I. (1997). Lewis X biosynthesis in *Helicobacter pylori*. Molecular cloning of an alpha(1,3)-fucosyltransferase gene. *Journal of Biological Chemistry.* **272**, 21349-56.

Martin, S. L., McColm, A. A. & Appelmelk, B. J. (2000). *H. pylori* adhesion and Lewis X. *Gastroenterology.* **119**, 1414-6.

Martin-Satue, M., Marrugat, R., Cancelas, J. A. & Blanco, J. (1998). Enhanced expression of alpha(1,3)-fucosyltransferase genes correlates with E-selectin-mediated adhesion and metastatic potential of human lung adenocarcinoma cells. *Cancer Research.* **58**, 1544-50.

Mattila, P., Räbinä, J., Hortling, S., Helin, J. & Renkonen, R. (2000). Functional expression of *Escherichia coli* enzymes synthesizing GDP-L-fucose from inherent GDP-D-mannose in *Saccharomyces cerevisiae*. *Glycobiology.* **10**, 1041-7.

McGowan, C. C., Necheva, A., Thompson, S. A., Cover, T. L. & Blaser, M. J. (1998). Acid-induced expression of an LPS-associated gene in *Helicobacter pylori*. *Molecular Microbiology.* **30**, 19-31.

Menon, S., Stahl, M., Kumar, R., Xu, G. Y. & Sullivan, F. (1999). Stereochemical course and steady state mechanism of the reaction catalyzed by the GDP-fucose synthetase from *Escherichia coli*. *Journal of Biological Chemistry.* **274**, 26743-50.

Mergaert, P., Van Montagu, M. & Holsters, M. (1997). The nodulation gene *nolK* of *Azorhizobium caulinodans* is involved in the formation of GDP-fucose from GDP-mannose. *FEBS Letters.* **409**, 312-6.

Molinaro, A., Silipo, A., Lanzetta, R., Newman, M. A., Dow, J. M. & Parrilli, M. (2003). Structural elucidation of the O-chain of the lipopolysaccharide from *Xanthomonas campestris* strain 8004. *Carbohydrate Research.* **338**, 277-81.

Monteiro, M. A., Appelmelk, B. J., Rasko, D. A., Moran, A. P., Hynes, S. O., MacLean, L. L., Chan, K. H., Michael, F. S., Logan, S. M., O'Rourke, J., Lee, A., Taylor, D. E. & Perry, M. B. (2000). Lipopolysaccharide structures of *Helicobacter pylori* genomic strains 26695 and J99, mouse model *H. pylori* Sydney strain, *H. pylori* P466 carrying sialyl Lewis X, and H. pylori UA915 expressing Lewis B classification of *H. pylori* lipopolysaccharides into glycotype families. *European Journal of Biochemistry.* **267**, 305-20.

Monteiro, M. A., Chan, K. H., Rasko, D. A., Taylor, D. E., Zheng, P. Y., Appelmelk, B. J., Wirth, H. P., Yang, M., Blaser, M. J., Hynes, S. O., Moran, A. P. & Perry, M. B. (1998). Simultaneous expression of type 1 and type 2 Lewis blood group antigens by *Helicobacter pylori* lipopolysaccharides. Molecular mimicry between *H. pylori* lipopolysaccharides and human gastric epithelial cell surface glycoforms. *Journal of Biological Chemistry.* **273**, 11533-43.

Moran, A. P., Knirel, Y. A., Senchenkova, S. N., Widmalm, G., Hynes, S. O. & Jansson, P. E. (2002). Phenotypic variation in molecular mimicry between *Helicobacter pylori* lipopolysaccharides and human gastric epithelial cell surface glycoforms. Acid-induced phase variation in Lewis(x) and Lewis(y) expression by *H. pylori* lipopolysaccharides. *Journal of Biological Chemistry.* **277**, 5785-95.

Moran, A. P., O'Malley, D. T., Kosunen, T. U. & Helander, I. M. (1994). Biochemical characterization of *Campylobacter fetus* lipopolysaccharides. *Infection & Immunity.* **62**, 3922-9.

Moran, A. P. & Prendergast, M. M. (2001). Molecular mimicry in *Campylobacter jejuni* and *Helicobacter pylori* lipopolysaccharides: contribution of gastrointestinal infections to autoimmunity. *Journal of Autoimmunity.* **16**, 241-56.

Mulichak, A. M., Bonin, C. P., Reiter, W. D. & Garavito, R. M. (2002). Structure of the MUR1 GDP-mannose 4,6-dehydratase from *Arabidopsis thaliana*: implications for ligand binding and specificity. *Biochemistry.* **41**, 15578-89.

Nakano, Y., Suzuki, N., Yoshida, Y., Nezu, T., Yamashita, Y. & Koga, T. (2000). Thymidine diphosphate-6-deoxy-L-*lyxo*-4-hexulose reductase synthesizing dTDP-6-deoxy-L-talose from *Actinobacillus actinomycetemcomitans*. *Journal of Biological Chemistry.* **275**, 6806-12.

Nedrud, J. G. (1999). Animal models for gastric *Helicobacter* immunology and vaccine studies. *FEMS Immunology & Medical Microbiology.* **24**, 243-50.

Niemelä, R., Natunen, J., Majuri, M. L., Maaheimo, H., Helin, J., Lowe, J. B., Renkonen, O. & Renkonen, R. (1998). Complementary acceptor and site specificities of Fuc-TIV and Fuc-TVII allow effective biosynthesis of sialyl-TriLex and related polylactosamines present on glycoprotein counterreceptors of selectins. *Journal of Biological Chemistry.* **273**, 4021-6.

Ohyama, C., Smith, P. L., Angata, K., Fukuda, M. N., Lowe, J. B. & Fukuda, M. (1998). Molecular cloning and expression of GDP-D-mannose-4,6-dehydratase, a key enzyme for fucose metabolism defective in Lec13 cells. *Journal of Biological Chemistry.* **273**, 14582-7.

Okazaki, R., Okazaki, T., Strominger, J. L. & Michelson, A. M. (1962). Thymidine diphosphate 4-keto-6-deoxy-D-glucose, an intermediate in thymidine diphosphate L-rhamnose synthesis in *Escherichia coli* strains. *Journal of Biological Chemistry.* **237**, 3014-26.

Oppermann, U. C., Filling, C. & Jörnvall, H. (2001). Forms and functions of human SDR enzymes. *Chemico-Bioligical Interactions.* **130-132**, 699-705.

Oriol, R., Mollicone, R., Cailleau, A., Balanzino, L. & Breton, C. (1999). Divergent evolution of fucosyltransferase genes from vertebrates, invertebrates, and bacteria. *Glycobiology.* **9**, 323-34.

Osaki, T., Yamaguchi, H., Taguchi, H., Fukuda, M., Kawakami, H., Hirano, H., Watanabe, S., Takagi, A. & Kamiya, S. (1998). Establishment and characterisation of a monoclonal antibody to inhibit adhesion of *Helicobacter pylori* to gastric epithelial cells. *Journal of Medical Microbiology.* **47**, 505-12.

Oths, P. J., Mayer, R. M. & Floss, H. G. (1990). Stereochemistry and mechanism of the GDP-mannose dehydratase reaction. *Carbohydrate Research.* **198**, 91-100.

Overton, K. & Serif, G. S. (1981). Synthesis of L-fucose in thyroid tissue. *Biochimica et Biophysica Acta.* **675**, 281-4.

Ovod, V., Rudolph, K., Knirel, Y. & Krohn, K. (1996). Immunochemical characterization of O polysaccharides composing the alpha-D-rhamnose backbone of lipopolysaccharide of *Pseudomonas syringae* and classification of bacteria into serogroups O1 and O2 with monoclonal antibodies. *Journal of Bacteriology.* **178**, 6459-65.

Packer, N. H., Lawson, M. A., Jardine, D. R. & Redmond, J. W. (1998). A general approach to desalting oligosaccharides released from glycoproteins. *Glycoconjugate Journal.* **15**, 737-47.

Palmieri, M. J., Berry, G. T., Player, D. A., Rogers, S. & Segal, S. (1991). The concentration of red blood cell UDPglucose and UDPgalactose determined by high-performance liquid chromatography. *Analytical Biochemistry.* **194**, 388-93.

Parkhill, J., Dougan, G., James, K. D., Thomson, N. R., Pickard, D., Wain, J., Churcher, C., Mungall, K. L., Bentley, S. D., Holden, M. T., Sebaihia, M., Baker, S., Basham, D., Brooks, K., Chillingworth, T., Connerton, P., Cronin, A., Davis, P., Davies, R. M., Dowd, L., White, N., Farrar, J., Feltwell, T., Hamlin, N., Haque, A., Hien, T. T., Holroyd, S., Jagels, K., Krogh, A., Larsen, T. S., Leather, S., Moule, S., O'Gaora, P., Parry, C., Quail, M., Rutherford, K., Simmonds, M., Skelton, J., Stevens, K., Whitehead, S. & Barrell, B. G. (2001). Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. *Nature.* **413**, 848-52.

Parkhill, J., Wren, B. W., Thomson, N. R., Titball, R. W., Holden, M. T., Prentice, M. B., Sebaihia, M., James, K. D., Churcher, C., Mungall, K. L., Baker, S., Basham, D., Bentley, S. D., Brooks, K., Cerdeno-Tarraga, A. M., Chillingworth, T., Cronin, A., Davies, R. M., Davis, P., Dougan, G., Feltwell, T., Hamlin, N., Holroyd, S., Jagels, K., Karlyshev, A. V., Leather, S., Moule, S., Oyston, P. C., Quail, M., Rutherford, K., Simmonds, M., Skelton, J., Stevens, K., Whitehead, S. & Barrell, B. G. (2001). Genome sequence of *Yersinia pestis*, the causative agent of plague. *Nature.* **413**, 523-7.

Perry, M. B., MacLean, L. M., Brisson, J. R. & Wilson, M. E. (1996). Structures of the antigenic O-polysaccharides of lipopolysaccharides produced by *Actinobacillus actinomycetemcomitans* serotypes a, c, d and e. *European Journal of Biochemistry.* **242**, 682-8.

Räbinä, J., Mattila, P. & Renkonen, R. (2000). Two time-resolved fluorometric high-throughput assays for quantitation of GDP-L-fucose. *Analytical Biochemistry.* **286**, 173-8.

Raetz, C. R. & Whitfield, C. (2002). Lipopolysaccharide endotoxins. *Annual Review of Biochemistry.* **71**, 635-700.

Rahim, R., Burrows, L. L., Monteiro, M. A., Perry, M. B. & Lam, J. S. (2000). Involvement of the *rml* locus in core oligosaccharide and O polysaccharide assembly in *Pseudomonas aeruginosa*. *Microbiology.* **146**, 2803-14.

Rahme, L. G., Ausubel, F. M., Cao, H., Drenkard, E., Goumnerov, B. C., Lau, G. W., Mahajan-Miklos, S., Plotnikova, J., Tan, M. W., Tsongalis, J., Walendziewicz, C. L. & Tompkins, R. G. (2000). Plants and animals share functionally common bacterial virulence factors. *Proceedings of the National Academy of Sciences of the United States of America* **. 97**, 8815-8821.

Rahme, L. G., Stevens, E. J., Wolfort, S. F., Shao, J., Tompkins, R. G. & Ausubel, F. M. (1995). Common virulence factors for bacterial pathogenicity in plants and animals. *Science* **268**, 1899-1902.

Rasko, D. A., Wang, G., Monteiro, M. A., Palcic, M. M. & Taylor, D. E. (2000). Synthesis of mono- and di-fucosylated type I Lewis blood group antigens by *Helicobacter pylori*. *European Journal of Biochemistry.* **267**, 6059-66.

Rasko, D. A., Wang, G., Palcic, M. M. & Taylor, D. E. (2000). Cloning and characterization of the alpha(1,3/4) fucosyltransferase of *Helicobacter pylori*. *Journal of Biological Chemistry.* **275**, 4988-94.

Reeves, P. R., Hobbs, M., Valvano, M. A., Skurnik, M., Whitfield, C., Coplin, D., Kido, N., Klena, J., Maskell, D., Raetz, C. R. & Rick, P. D. (1996). Bacterial polysaccharide synthesis and gene nomenclature. *Trends in Microbiology.* **4**, 495-503.

Rizzi, M., Tonetti, M., Vigevani, P., Sturla, L., Bisso, A., Flora, A. D., Bordo, D. & Bolognesi, M. (1998). GDP-4-keto-6-deoxy-D-mannose epimerase/reductase from *Escherichia coli*, a key enzyme in the biosynthesis of GDP-L-fucose, displays the structural characteristics of the RED protein homology superfamily. *Structure.* **6**, 1453-65.

Rocchetta, H. L., Burrows, L. L. & Lam, J. S. (1999). Genetics of O-antigen biosynthesis in *Pseudomonas aeruginosa*. *Microbiology & Molecular Biology Reviews.* **63**, 523-53.

Rocchetta, H. L., Burrows, L. L., Pacan, J. C. & Lam, J. S. (1998). Three rhamnosyltransferases responsible for assembly of the A-band D-rhamnan polysaccharide in *Pseudomonas aeruginosa*: a fourth transferase, WbpL, is required for the initiation of both A-band and B-band lipopolysaccharide synthesis. *Molecular Microbiology.* **28**, 1103-1119.

Rocchetta, H. L., Pacan, J. C. & Lam, J. S. (1998). Synthesis of the A-band polysaccharide sugar D-rhamnose requires Rmd and WbpW: identification of multiple AlgA homologues, WbpW and ORF488, in *Pseudomonas aeruginosa*. *Molecular Microbiology.* **29**, 1419-1434.

Romanos, M. A., Scorer, C. A. & Clare, J. J. (1992) Foreign gene expression in yeast: a review. *Yeast.* 8, 423-488.

Roos, C., Kolmer, M., Mattila, P. & Renkonen, R. (2002). Composition of *Drosophila melanogaster* proteome involved in fucosylated glycan metabolism. *Journal of Biological Chemistry.* **277**, 3168-75.

Rosano, C., Bisso, A., Izzo, G., Tonetti, M., Sturla, L., De Flora, A. & Bolognesi, M. (2000). Probing the catalytic mechanism of GDP-4-keto-6-deoxy-D-mannose Epimerase/Reductase by kinetic and crystallographic characterization of site-specific mutants. *Journal of Molecular Biology.* **303**, 77-91.

Rush, J. S. & Waechter, C. J. (1995). Method for the determination of cellular levels of guanosine-5'-diphosphate-mannose based on a weak interaction with concanavalin A at low pH. *Analytical Biochemistry.* **224**, 494-501.

Russa, R., Urbanik-Sypniewska, T., Lindstrom, K. & Mayer, H. (1995). Chemical characterization of two lipopolysaccharide species isolated from *Rhizobium loti* NZP2213. *Archives of Microbiology.* **163**, 345-51.

Saarela, M., Asikainen, S., Alaluusua, S., Pyhälä, L., Lai, C. H. & Jousimies-Somer, H. (1992). Frequency and stability of mono- or poly-infection by *Actinobacillus actinomycetemcomitans* serotypes a, b, c, d or e. *Oral Microbiology and Immunology.* **7**, 277-9.

Sambrook, J. & Russell, D. W. (2001). *Molecular cloning. A laboratory manual. 3th Ed.* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Senchenkova, S. N., Shashkov, A. S., Knirel, Y. A., McGovern, J. J. & Moran, A. P. (1996). The O-specific polysaccharide chain of *Campylobacter fetus* serotype B lipopolysaccharide is a D-rhamnan terminated with 3-O-methyl-D-rhamnose (D-acofriose). *European Journal of Biochemistry.* **239**, 434-8.

Senchenkova, S. N., Shashkov, A. S., Moran, A. P., Helander, I. M. & Knirel, Y. A. (1995). Structures of the O-specific polysaccharide chains of *Pectinatus cerevisiiphilus* and *Pectinatus frisingensis* lipopolysaccharides. *European Journal of Biochemistry.* **232**, 552-7.

Shibuya, N., Amano, K., Azuma, J., Nishihara, T., Kitamura, Y., Noguchi, T. & Koga, T. (1991). 6-Deoxy-D-talan and 6-deoxy-L-talan. Novel serotype-specific polysaccharide antigens from *Actinobacillus actinomycetemcomitans*. *Journal of Biological Chemistry.* **266**, 16318-23.

Simoons-Smit, I. M., Appelmelk, B. J., Verboom, T., Negrini, R., Penner, J. L., Aspinall, G. O., Moran, A. P., Fei, S. F., Shi, B. S., Rudnica, W., Savio, A. & de Graaff, J. (1996). Typing of *Helicobacter pylori* with monoclonal antibodies against Lewis antigens in lipopolysaccharide. *Journal of Clinical Microbiology.* **34**, 2196-200.

Skurnik, M. & Zhang, L. (1996). Molecular genetics and biochemistry of *Yersinia* lipopolysaccharide. *Apmis.* **104**, 849-72.

Smith, P. L., Myers, J. T., Rogers, C. E., Zhou, L., Petryniak, B., Becker, D. J., Homeister, J. W. & Lowe, J. B. (2002). Conditional control of selectin ligand expression and global fucosylation events in mice with a targeted mutation at the FX locus. *Journal of Cell Biology.* **158**, 801-15.

Somers, W. S., Stahl, M. L. & Sullivan, F. X. (1998). GDP-fucose synthetase from *Escherichia coli*: structure of a unique member of the short-chain dehydrogenase/reductase family that catalyzes two distinct reactions at the same active site. *Structure.* **6**, 1601-12.

Somoza, J. R., Menon, S., Schmidt, H., Joseph-McCarthy, D., Dessen, A., Stahl, M. L., Somers, W. S. & Sullivan, F. X. (2000). Structural and kinetic analysis of *Escherichia coli* GDP-mannose 4,6 dehydratase provides insights into the enzyme's catalytic mechanism and regulation by GDP-fucose. *Structure.* **8**, 123-35.

Sosroseno, W. & Herminajeng, E. (2002). The role of macrophages in the induction of murine immune response to *Actinobacillus actinomycetemcomitans*. *Journal of Medical Microbiology.* **51**, 581-8.

Stevenson, G., Lan, R. & Reeves, P. R. (2000). The colanic acid gene cluster of *Salmonella enterica* has a complex history. *FEMS Microbiology Letters.* **191**, 11-6.

Stotland, P. K., Radzioch, D. & Stevenson, M. M. (2000). Mouse models of chronic lung infection with *Pseudomonas aeruginosa*: models for the study of cystic fibrosis. *Pediatric Pulmonology.* **30**, 413-24.

Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E., Lory, S. & Olson, M. V. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature.* **406**, 959-64.

Sturla, L., Bisso, A., Zanardi, D., Benatti, U., De Flora, A. & Tonetti, M. (1997). Expression, purification and characterization of GDP-D-mannose 4,6-dehydratase from *Escherichia coli*. *FEBS Letters.* **412**, 126-30.

Sullivan, F. X., Kumar, R., Kriz, R., Stahl, M., Xu, G. Y., Rouse, J., Chang, X. J., Boodhoo, A., Potvin, B. & Cumming, D. A. (1998). Molecular cloning of human GDP-mannose 4,6-dehydratase and reconstitution of GDP-fucose biosynthesis i*n vitro*. *Journal of Biological Chemistry.* **273**, 8193-202.

Suzuki, N., Nakano, Y., Yoshida, Y., Nakao, H., Yamashita, Y. & Koga, T. (2000). Genetic analysis of the gene cluster for the synthesis of serotype a-specific polysaccharide antigen in *Actinobacillus actinomycetemcomitans*. *Biochimica et Biophysica Acta.* **1517**, 135-8.

Suzuki, N., Nakano, Y., Yoshida, Y., Nezu, T., Terada, Y., Yamashita, Y. & Koga, T. (2002). Guanosine diphosphate-4-keto-6-deoxy-D-mannose reductase in the pathway for the synthesis of GDP-6-deoxy-D-talose in *Actinobacillus actinomycetemcomitans*. *European Journal of Biochemistry.* **269**, 5963-71.

Tan, M. W., Mahajan-Miklos, S. & Ausubel, F. M. (1999). Killing of *Caenorhabditis elegans* by *Pseudomonas aeruginosa* used to model mammalian bacterial pathogenesis. *Proceedings of the National Academy of Sciences of the United States of America.* **96**, 715-720.

Thoden, J. B., Frey, P. A. & Holden, H. M. (1996). High-resolution X-ray structure of UDP-galactose 4-epimerase complexed with UDP-phenol. *Protein Science.* **5**, 2149-61.

Todorow, Z., Spang, A., Carmack, E., Yates, J. & Schekman, R. (2000). Active recycling of yeast Golgi mannosyltransferase complexes through the endoplasmic reticulum. *Proceedings of the National Academy of Sciences of the United States of America.* **97**, 13643-13648.

Toivonen, S., Nishihara, S., Narimatsu, H., Renkonen, O. & Renkonen, R. (2002). Fuc-TIX: a versatile alpha1,3-fucosyltransferase with a distinct acceptor- and site-specificity profile. *Glycobiology.* **12**, 361-8.

Tomb, J. F., White, O., Kerlavage, A. R., Clayton, R. A., Sutton, G. G., Fleischmann, R. D., Ketchum, K. A., Klenk, H. P., Gill, S., Dougherty, B. A., Nelson, K., Quackenbush, J., Zhou, L., Kirkness, E. F., Peterson, S., Loftus, B., Richardson, D., Dodson, R., Khalak, H. G., Glodek, A., McKenney, K., Fitzegerald, L. M., Lee, N., Adams, M. D., Venter, J. C. & *et al*. (1997). The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature.* **388**, 539-47.

Tomiya, N., Ailor, E., Lawrence, S. M., Betenbaugh, M. J. & Lee, Y. C. (2001). Determination of nucleotides and sugar nucleotides involved in protein glycosylation by high-performance anion-exchange

chromatography: sugar nucleotide contents in cultured insect cells and mammalian cells. *Analytical Biochemistry.* **293**, 129-37.

Tonetti, M., Rizzi, M., Vigevani, P., Sturla, L., Bisso, A., De Flora, A. & Bolognesi, M. (1998). Preliminary crystallographic investigations of recombinant GDP-4-keto-6-deoxy-D-mannose epimerase/reductase from *E. coli*. *Acta Crystallographica Section D-Biological Crystallography.* **54**, 684-6.

Tonetti, M., Sturla, L., Bisso, A., Benatti, U. & De Flora, A. (1996). Synthesis of GDP-L-fucose by the human FX protein. *Journal of Biological Chemistry.* **271**, 27274-9.

Tonetti, M., Sturla, L., Bisso, A., Zanardi, D., Benatti, U. & De Flora, A. (1998). The metabolism of 6-deoxyhexoses in bacterial and animal cells. *Biochimie.* **80**, 923-31.

Toppila, S., Paavonen, T., Laitinen, A., Laitinen, L. A. & Renkonen, R. (2000). Endothelial sulfated sialyl Lewis x glycans, putative L-selectin ligands, are preferentially expressed in bronchial asthma but not in other chronic inflammatory lung diseases. *American Journal of Respiratory Cell & Molecular Biology.* **23**, 492-8.

Toppila, S., Paavonen, T., Nieminen, M. S., Hayry, P. & Renkonen, R. (1999). Endothelial L-selectin ligands are likely to recruit lymphocytes into rejecting human heart transplants. *American Journal of Pathology.* **155**, 1303-10.

Trefzer, A., Salas, J. A. & Bechthold, A. (1999). Genes and enzymes involved in deoxysugar biosynthesis in bacteria. *Natural Product Reports.* **16**, 283-99.

Unligil, U. M. & Rini, J. M. (2000). Glycosyltransferase structure and mechanism. *Current Opinion in Structural Biology.* **10**, 510-7.

Ura, H., Denno, R., Hirata, K., Yamaguchi, K., Yasoshima, T. & Shishido, T. (1997). Close correlation between increased sialyl-Lewis x expression and metastasis in human gastric carcinoma. *World Journal of Surgery.* **21**, 773-6.

van Putten, J. P. (1993). Phase variation of lipopolysaccharide directs interconversion of invasive and immuno-resistant phenotypes of *Neisseria gonorrhoeae*. *EMBO Journal.* **12**, 4043-51.

Wang, G., Boulton, P. G., Chan, N. W., Palcic, M. M. & Taylor, D. E. (1999). Novel *Helicobacter pylori* alpha1,2-fucosyltransferase, a key enzyme in the synthesis of Lewis antigens. *Microbiology.* **145**, 3245-53.

Wang, G., Ge, Z., Rasko, D. A. & Taylor, D. E. (2000). Lewis antigens in *Helicobacter pylori*: biosynthesis and phase variation. *Molecular Microbiology.* **36**, 1187-96.

Wang, G., Rasko, D. A., Sherburne, R. & Taylor, D. E. (1999). Molecular genetic basis for the variable expression of Lewis Y antigen in *Helicobacter pylori*: analysis of the alpha (1,2) fucosyltransferase gene. *Molecular Microbiology.* **31**, 1265-74.

Wang, L. & Reeves, P. R. (1998). Organization of *Escherichia coli* O157 O antigen gene cluster and identification of its specific genes. *Infection & Immunity.* **66**, 3545-51.

Wang, L. & Reeves, P. R. (2000). The *Escherichia coli* O111 and *Salmonella enterica* O35 gene clusters: gene clusters encoding the same colitose-containing O antigen are highly conserved. *Journal of Bacteriology.* **182**, 5256-61.

Wang, Y., Shao, L., Shi, S., Harris, R. J., Spellman, M. W., Stanley, P. & Haltiwanger, R. S. (2001). Modification of epidermal growth factor-like repeats with O-fucose. Molecular cloning and expression of a novel GDP-fucose protein O-fucosyltransferase. *Journal of Biological Chemistry.* **276**, 40338-45.

Weckesser, J., Mayer, H. & Fromme, I. (1973). O-methyl sugars in lipopolysaccharides of *Rhodospirillaceae*. Identification of 3-O-methyl-D-mannose in *Rhodopseudomonas viridis* and of 4-O-methyl-D-xylose and 3-O-methyl-6-deoxy-D-talose in *Rhodopseudomonas palustris* respectively. *Biochemical Journal.* **135**, 293-7.

Weiser, J. N. & Pan, N. (1998). Adaptation of *Haemophilus influenzae* to acquired and innate humoral immunity based on phase variation of lipopolysaccharide. *Molecular Microbiology.* **30**, 767-75.

Whitfield, C. & Roberts, I. S. (1999). Structure, assembly and regulation of expression of capsules in *Escherichia coli*. *Molecular Microbiology.* **31**, 1307-19.

Villeneuve, S., Souchon, H., Riottot, M. M., Mazie, J. C., Lei, P., Glaudemans, C. P., Kovac, P., Fournier, J. M. & Alzari, P. M. (2000). Crystal structure of an anti-carbohydrate antibody directed against *Vibrio cholerae* O1 in complex with antigen: molecular basis for serotype specificity. *Proceedings of the National Academy of Sciences of the United States of America.* **97**, 8433-8.

Winn, A. M. & Wilkinson, S. G. (1998). The O7 antigen of *Stenotrophomonas maltophilia* is a linear D-rhamnan with a trisaccharide repeating unit that is also present in polymers for some *Pseudomonas* and *Burkholderia* species. *FEMS Microbiology Letters.* **166**, 57-61.

Vinogradov, E. V., Campos-Portuguez, S., Yokota, A. & Mayer, H. (1994). The structure of the O-specific polysaccharide from *Thiobacillus ferrooxidans* IFO 14262. *Carbohydrate Research.* **261**, 103-9.

Wirth, H. P., Yang, M., Karita, M. & Blaser, M. J. (1996). Expression of the human cell surface glycoconjugates Lewis x and Lewis y by *Helicobacter pylori* isolates is related to cagA status. *Infection & Immunity.* **64**, 4598-605.

Wu, B., Zhang, Y. & Wang, P. G. (2001). Identification and characterization of GDP-D-mannose 4,6-dehydratase and GDP-L-fucose snthetase in a GDP-L-fucose biosynthetic gene cluster from *Helicobacter pylori*. *Biochemical & Biophysical Research Communications.* **285**, 364-71.

Wu, B., Zhang, Y., Zheng, R., Guo, C. & Wang, P. G. (2002). Bifunctional phosphomannose isomerase/GDP-D-mannose pyrophosphorylase is the point of control for GDP-D-mannose biosynthesis in *Helicobacter pylori*. *FEBS Letters.* **519**, 87-92.

Xu, Y., Singh, K. V., Qin, X., Murray, B. E. & Weinstock, G. M. (2000). Analysis of a gene cluster of *Enterococcus faecalis* involved in polysaccharide biosynthesis. *Infection & Immunity.* **68**, 815-23.

Yamamoto, K., Katayama, I., Onoda, Y., Inami, M., Kumagai, H. & Tochikura, T. (1993). Evidence that the enzyme catalyzing the conversion of guanosine diphosphate D-mannose to a 4-keto sugar nucleotide intermediate requires nicotinamide adenine dinucleotide phosphate. *Archives of Biochemistry & Biophysics.* **300**, 694-8.

Yamashita, Y., Tomihisa, K., Nakano, Y., Shimazaki, Y., Oho, T. & Koga, T. (1999). Recombination between gtfB and gtfC is required for survival of a dTDP-rhamnose synthesis-deficient mutant of *Streptococcus mutans* in the presence of sucrose. *Infection & Immunity.* **67**, 3693-7.

Yokota, S., Kaya, S., Sawada, S., Kawamura, T., Araki, Y. & Ito, E. (1987). Characterization of a polysaccharide component of lipopolysaccharide from *Pseudomonas aeruginosa* IID 1008 (ATCC 27584) as D-rhamnan. *European Journal of Biochemistry.* **167**, 203-9.

Yoshida, Y., Nakano, Y., Nezu, T., Yamashita, Y. & Koga, T. (1999). A novel NDP-6-deoxyhexosyl-4-ulose reductase in the pathway for the synthesis of thymidine diphosphate-D-fucose. *Journal of Biological Chemistry.* **274**, 16933-9.

Zähringer, U., Rettenmaier, H., Moll, H., Senchenkova, S. N. & Knirel, Y. A. (1997). Structure of a new 6-deoxy-alpha-D-talan from *Burkholderia (Pseudomonas) plantarii* strain DSM 6535, which is different from the O-chain of the lipopolysaccharide. *Carbohydrate Research* **300**, 143-51.

Zambon, J. J., Slots, J. & Genco, R. J. (1983). Serology of oral *Actinobacillus actinomycetemcomitans* and serotype distribution in human periodontal disease. *Infection & Immunity.* **41**, 19-27.

Zhang, L., Li, X. Z. & Poole, K. (2000). Multiple antibiotic resistance in *Stenotrophomonas maltophilia*: involvement of a multidrug efflux system. *Antimicrobial Agents & Chemotherapy.* **44**, 287-93.

Zhang, L., Toivanen, P. & Skurnik, M. (1996). The gene cluster directing O-antigen biosynthesis in *Yersinia enterocolitica* serotype 0:8: identification of the genes for mannose and galactose biosynthesis and the gene for the O-antigen polymerase. *Microbiology.* **142**, 277-88.