The structure, composition, and application of the cell envelope from *Caulobacter crescentus*

by

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Abstract

This document provides brief instructions for using the `ubcdiss` class to write a UBC-conformant dissertation in LaTeX. This document is itself written using the `ubcdiss` class and is intended to serve as an example of writing a dissertation in LaTeX. This document has embedded Unique Resource Locators (URLS) and is intended to be viewed using a computer-based Portable Document Format (PDF) reader.

Note: Abstracts should generally try to avoid using acronyms.

Note: at University of British Columbia (UBC), both the Graduate and Postdoctoral Studies (GPS) Ph.D. defence programme and the Library's online submission system restricts abstracts to 350 words.

Preface

At UBC, a preface may be required. Be sure to check the GPS guidelines as they may have specific content to be included.

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Glossary

This glossary uses the handy `acroynym` package to automatically maintain the glossary. It uses the package's `printonlyused` option to include only those acronyms explicitly referenced in the LaTeX source.

DOI	Document Object Identifier (see http://doi.org)
GPS	Graduate and Postdoctoral Studies
PDF	Portable Document Format
RCS	Revision control system, a software tool for tracking changes to a set of files
URL	Unique Resource Locator, used to describe a means for obtaining some resource on the world wide web
LPS	lipopolysaccharide
PS	polysaccharide
OPS	O-specific polysaccharide
OS	oligosaccharide
UV	ultraviolet Light
MALDI-TOF	matrix assisted laser desorption/ionization-time of flight mass spectroscopy
S-LAYER	protein surface layer
GC-MS	gas chromatography-mass spectroscopy
NMR	nuclear magnetic resonance spectroscopy
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
EDTA	ethylenediaminetetraacetic acid
COSY	correlation spectroscopy

GCOSY	gradient correlation spectroscopy
TOCSY	total correlation spectroscopy
ROESY	rotating frame nuclear Overhauser effect spectroscopy
HSQC	heteronuclear single quantum coherence
GHSQC	gradient heteronuclear single quantum coherence
HMBC	heteronuclear multiple bond coherence
GHMBC	gradient heteronuclear multiple bond coherence
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect spectroscopy
HMQC	heteronuclear multiple-quantum correlation spectroscopy
TLC	thin-layer chromatography
ABC	ATP-binding cassette
EPS	extracellular polysaccharide
ESI	electrospray ionization
TFA	trifluoroacetic acid

Acknowledgments

Thank those people who helped you.

Don’t forget your parents or loved ones.

You may wish to acknowledge your funding sources.

Chapter 1

Introduction

If I have seen farther it is by standing on the shoulders of Giants. — Sir Isaac Newton (1855)

This document provides a quick[1] set of instructions for using the `ubcdiss` class to write a dissertation in LaTeX. Unfortunately this document cannot provide an introduction to using LaTeX. The classic reference for learning LaTeX is Lamport's book.[2] There are also many freely-available tutorials online;

seems to be excellent. The source code for this docment, however, is intended to serve as an example for creating a LaTeX version of your dissertation.

We start by discussing organizational issues, such as splitting your dissertation into multiple files, in section 1.1. We then cover the ease of managing cross-references in LaTeX in section 1.2. We cover managing and using bibliographies with BibTeX in section 1.3. We briefly describe typesetting attractive tables in section 1.4. We briefly describe including external figures in section 1.5, and using special characters and symbols in section 1.6. As it is often useful to track different versions of your dissertation, we discuss revision control further in section 1.8. We conclude with pointers to additional sources of information in section 1.10.

1.1 Suggested Thesis Organization

The University of British Columbia (UBC) Graduate and Postdoctoral Studies (GPS) specifies a partfoocular arrangement of the components forming a thesis. This template reflects that arrangement.

In terms of writing your thesis, the recommended best practice for organizing large documents in LaTeX is to place each chapter in a separate file. These chapters are then included from the main file through the use of `\include{file}`. A thesis might be described as six files such as `intro.tex`, `relwork.tex`, `model.tex`, `eval.tex`, `discuss.tex`, and `concl.tex`.

We also encourage you to use macros for separating how something will be typeset (bold, or italics) from the meaning of that something. For example, if you look at `intro.tex`, you will see repeated uses of a macro `\file{}` to indicate file names. The `\file{}` macro is defined in the file `macros.tex`.

[1] Michael D Jones et al. *Arth. Rheu.*, **62**: 2726–2735, 2010.

[2] Leslie Lamport. *LaTeX: A Document Preparation System*. 2nd ed. Addison-Wesley, 1994.

The consistent use of `\file{}` throughout the text not only indicates that the argument to the macro represents a file (providing meaning or semantics), but also allows easily changing how file names are typeset simply by changing the definition of the `\file{}` macro. `macros.tex` contains other useful macros for properly typesetting things like the proper uses of the latinate *exempli gratiā* and *id est* (`\eg` and `\ie`), web references with a footnoted URL (`\webref{url}{text}`), as well as definitions specific to this documentation (`\latexpackage{}`).

1.2 Making Cross-References

LaTeX make managing cross-references easy, and the `hyperref` package's `\cref{}` command makes it easier still.

A thing to be cross-referenced, such as a section, figure, or equation, is *labelled* using a unique, user-provided identifier, defined using the `\label{}` command. The thing is referenced elsewhere using the `\cref{}` command. For example, this section was defined using:

> We then cover the ease of managing cross-references in LaTeX in section 1.2.

The label is any simple sequence of characters, numbers, digits, and some punctuation marks such as ":" and "–"; there should be no spaces. Try to use a consistent key format: this simplifies remembering how to make references. This document uses a prefix to indicate the type of the thing being referenced, such as `sec` for sections, `fig` for figures, `tbl` for tables, and `eqn` for equations.

For details on defining the text used to describe the type of *thing*, search `diss.tex` and the `hyperref` documentation for `crefname`.

1.3 Managing Bibliographies with BibTeX

One of the primary benefits of using LaTeX is its companion program, BibTeX, for managing bibliographies and citations. Managing bibliographies has three parts: (i) describing references, (ii) citing references, and (iii) formatting cited references.

1.3.1 Describing References

BibTeX defines a standard format for recording details about a reference. These references are recorded in a file with a `.bib` extension. BibTeX supports a broad range of references, such as books, articles, items in a conference proceedings, chapters, technical reports, manuals, dissertations, and unpublished manuscripts. A reference may include attributes such as the authors, the title, the page numbers, the Document Object Identifier (DOI), or a Unique Resource Locator (URL). A reference can also be augmented with personal attributes, such as a rating, notes, or keywords.

Each reference must be described by a unique *key*. A key is a simple sequence of characters, numbers, digits, and some punctuation marks such as ":" and "–"; there should be no spaces. A consistent key format simiplifies remembering how to make references. For example:

last-name–*year*–*contracted-title*

where *last-name* represents the last name for the first author, and *contracted-title* is some meaningful contraction of the title. Then Kiczales et al.'s seminal article on aspect-oriented programming [3] (published in 1997) might be given the key `kiczales-1997-aop`.

An example of a BibTEX `.bib` file is included as `biblio.bib`. A description of the format a `.bib` file is beyond the scope of this document. We instead encourage you to use one of the several reference managers that support the BibTEX format such as

These front ends are similar to reference manages such as EndNote or RefWorks.

1.3.2 Citing References

Having described some references, we then need to cite them. We do this using a form of the `\cite` command. For example:

When processed, the `\citet` will cause the paper's authors and a standardized reference to the paper to be inserted in the document, and will also include a formatted citation for the paper in the bibliography. For example:

present examples of crosscutting from programs written in several languages.

There are several forms of the `\cite` command (provided by the `natbib` package), as demonstrated in table 1.1. Note that the form of the citation (numeric or author-year) depends on the bibliography style (described in the next section). The `\citet` variant is used when the author names form an object in the sentence, whereas the `\citep` variant is used for parenthetic references, more like an end-note.

1.3.3 Formatting Cited References

BibTEX separates the citing of a reference from how the cited reference is formatted for a bibliography, specified with the `\bibliographystyle` command. There are many varieties, such as `plainnat`, `abbrvnat`, `unsrtnat`, and `vancouver`. This document was formatted with `abbrvnat`. Look through your TEX distribution for `.bst` files. Note that use of some `.bst` files do not emit all the information necessary to properly use `\citet{}`, `\citep{}`, `\citeyear{}`, and `\citeauthor{}`.

There are also packages available to place citations on a per-chapter basis (`bibunits`), as footnotes (`footbib`), and inline (`bibentry`). Those who wish to exert maximum control over their bibliography style should see the amazing `custom-bib` package.

Table 1.1: Available `cite` variants; the exact citation style depends on whether the bibliography style is numeric or author-year.

Variant	Result
`\cite`	Parenthetical citation (*e.g.* "[3]" or "(Kiczales et al. 1997)")
`\citet`	Textual citation: includes author (*e.g.* or or "Kiczales et al. (1997)")
`\citet*`	Textual citation with unabbreviated author list
`\citealt`	Like `\citet` but without parentheses
`\citep`	Parenthetical citation (*e.g.* "[3]" or "(Kiczales et al. 1997)")
`\citep*`	Parenthetical citation with unabbreviated author list
`\citealp`	Like `\citep` but without parentheses
`\citeauthor`	Author only (*e.g.* "Kiczales et al.")
`\citeauthor*`	Unabbreviated authors list (*e.g.* "Kiczales et al.")
`\citeyear`	Year of citation (*e.g.* "1997")

1.4 Typesetting Tables

made one grievous mistake in LaTeX: his suggested manner for typesetting tables produces typographic abominations. These suggestions have unfortunately been replicated in most LaTeX tutorials. These abominations are easily avoided simply by ignoring his examples illustrating the use of horizontal and vertical rules (specifically the use of `\hline` and `|`) and using the `booktabs` package instead.

The `booktabs` package helps produce tables in the form used by most professionally-edited journals through the use of three new types of dividing lines, or *rules*. Tables 1.1 and 1.2 are two examples of tables typeset with the `booktabs` package. The `booktabs` package provides three new commands for producing rules: `\toprule` for the rule to appear at the top of the table, `\midrule` for the middle rule following the table header, and `\bottomrule` for the bottom-most at the end of the table. These rules differ by their weight (thickness) and the spacing before and after. A table is typeset in the following manner:

See the `booktabs` documentation for advice in dealing with special cases, such as subheading rules, introducing extra space for divisions, and interior rules.

1.5 Figures, Graphics, and Special Characters

Most LaTeX beginners find figures to be one of the more challenging topics. In LaTeX, a figure is a *floating element*, to be placed where it best fits. The user is not expected to concern him/herself with the placement of the figure. The figure should instead be labelled, and where the figure is used, the text should use `\cref`

Figure 1.1: Proof of LaTeX's amazing abilities

Table 1.2: Useful LaTeX symbols

LaTeX	Result	LaTeX	Result
`\texttrademark`	™	`\&`	&
`\textcopyright`	©	`\{ \}`	{ }
`\textregistered`	®	`\%`	%
`\textsection`	§	`\verb!~!`	~
`\textdagger`	†	`\$`	$
`\textdaggerdbl`	‡	`\^{}`	^
`\textless`	<	`\_`	_
`\textgreater`	>		

to reference the figure's label. fig. 1.1 is an example of a figure. A figure is generally included as follows: There are three items of note:

1. External files are included using the `\includegraphics` command. This command is defined by the `graphicx` package and can often natively import graphics from a variety of formats. The set of formats supported depends on your TeX command processor. Both `pdflatex` and `xelatex`, for example, can import GIF, JPG, and PDF. The plain version of `latex` only supports EPS files.

2. The `\caption` provides a caption to the figure. This caption is normally listed in the List of Figures; you can provide an alternative caption for the LoF by providing an optional argument to the `\caption` like so:

   GPS generally prefers shortened single-line captions in the LoF: multiple-line captions are a bit unwieldy.

3. The `\label` command provides for associating a unique, user-defined, and descriptive identifier to the figure. The figure can be can be referenced elsewhere in the text with this identifier as described in section 1.2.

See Keith Reckdahls excellent guide for more details, *Using imported graphics in LaTeX2e*.

1.6 Special Characters and Symbols

1.7 Changing Page Widths and Heights

The `ubcdiss` class is based on the standard LaTeX `book` class that selects a line-width to carry approximately 66 characters per line. This character density is claimed to have a pleasing appearance and also supports more rapid reading [4]. I would recommend that you not change the line-widths!

1.7.1 The `geometry` Package

Some students are unfortunately saddled with misguided supervisors or committee members whom believe that documents should have the narrowest margins possible. The `geometry` package is helpful in such cases. Using this package is as simple as:

You should check the package's documentation for more complex uses.

1.7.2 Changing Page Layout Values By Hand

There are some miserable students with requirements for page layouts that vary throughout the document. Unfortunately the `geometry` can only be specified once, in the document's preamble. Such miserable students must set LaTeX's layout parameters by hand:

These settings necessarily require assuming a particular page height and width; in the above, the setting for `\textwidth` assumes a US Letter with an 8.5" width. The `geometry` package simply uses the page height and other specified values to derive the other layout values. The `layout` package provides a handy `\layout` command to show the current page layout parameters.

1.7.3 Making Temporary Changes to Page Layout

There are occasions where it becomes necessary to make temporary changes to the page width, such as to accomodate a larger formula. The `chngpage` package provides an `adjustwidth` environment that does just this. For example:

1.8 Keeping Track of Versions with Revision Control

Software engineers have used Revision control system (RCS) to track changes to their software systems for decades. These systems record the changes to the source code along with context as to why the change was required. These systems also support examining and reverting to particular revisions from their system's past.

An RCS can be used to keep track of changes to things other than source code, such as your dissertation. For example, it can be useful to know exactly which revision of your dissertation was sent to a particular committee member. Or to recover an accidentally deleted file, or a badly modified image. With a revision control system, you can tag or annotate the revision of your dissertation that was sent to your committee, or when you incorporated changes from your supervisor.

Unfortunately current revision control packages are not yet targetted to non-developers. But the Subversion project's

has greatly simplified using the Subversion revision control system for Windows users. You should consult your local geek.

A simpler alternative strategy is to create a GoogleMail account and periodically mail yourself zipped copies of your dissertation.

1.9 Recommended Packages

The real strength to LaTeX is found in the myriad of free add-on packages available for handling special formatting requirements. In this section we list some helpful packages.

1.9.1 Typesetting

enumitem: Supports pausing and resuming enumerate environments.

ulem: Provides two new commands for striking out and crossing out text (`\sout{text}` and `\xout{text}` respectively) The package should likely be used as follows:

```
\usepackage[normalem,normalbf]{ulem}
```

to prevent the package from redefining the emphasis and bold fonts.

chngpage: Support changing the page widths on demand.

mhchem: Support for typesetting chemical formulae and reaction equations.

Although not a package, the
command is very useful for creating changebar'd versions of your dissertation.

1.9.2 Figures, Tables, and Document Extracts

pdfpages: Insert pages from other PDF files. Allows referencing the extracted pages in the list of figures, adding labels to reference the page from elsewhere, and add borders to the pages.

subfig: Provides for including subfigures within a figure, and includes being able to separately reference the subfigures. This is a replacement for the older `subfigure` environment.

rotating: Provides two environments, sidewaystable and sidewaysfigure, for typesetting tables and figures in landscape mode.

longtable: Support for long tables that span multiple pages.

tabularx: Provides an enhanced tabular environment with auto-sizing columns.

ragged2e: Provides several new commands for setting ragged text (*e.g.* forms of centered or flushed text) that can be used in tabular environments and that support hyphenation.

1.9.3 Bibliography Related Packages

bibunits: Support having per-chapter bibliographies.

footbib: Cause cited works to be rendered using footnotes.

bibentry: Support placing the details of a cited work in-line.

custom-bib: Generate a custom style for your bibliography.

1.10 Moving On

At this point, you should be ready to go. Other handy web resources:

Chapter 2

The core and O-polysaccharide structure of the *Caulobacter crescentus* lipopolysaccharide

2.1 Introduction

Caulobacter crescentus is an aquatic alphaproteobacterium well known for a stalked, crescent cell morphology, asymmetric cell division, and a protein surface layer (S-LAYER). *C. crescentus* is a widely studied model organism for cell development and differentiation; despite this, the structure of its (lipopolysaccharide (LPS)) has not yet been fully determined.

Interest in the LPS of *C. crescentus* is focused on its immunological profile[5] and its structural role as an anchor for the self-assembled, paracrystalline S-LAYER.[6] The LPS of *C. crescentus* possesses a much reduced immunogenic activity, most likely due to its lipid A structure, which is significantly different from that of LPS from enteric bacteria. The lipid A structure has been reported;[5] it is a unique molecule containing a di-diaminoglucose backbone (instead of di-glucosamine) and two galacturonate moieties that replace the canonical phosphates that are on each end of the disaccharide in most lipid A molecules. The *C. crescentus* S-LAYER non-covalently attaches to the O-specific polysaccharide (OPS).[6] However, the OPS structure has not been resolved. Genetic analyses have pointed towards the unusual N-acetylperosamine being a major component.[7] A notable feature of this O-antigen is that it exists completely hidden beneath the S-layer, presumably inaccessible to the environment.[6] Carbohydrate structures from non-pathogenic bacterial LPS are rarely studied and an LPS that is sequestered beneath an S-LAYER is not represented in the literature.

In the present study our data has determined the core oligosaccharide structure from *C. crescentus* CB15 NA1000, (advancing an earlier report of core composition[8]) as well as the central backbone and non-reducing ends of its OPS. Unexpectedly, we identified a previously unknown rhamnan polysaccharide. Along with previous reports on lipid A[5] and extracellular polysaccharide (EPS),[9] we believe the major

[5] John Smit et al. *Innate Immun*, **14**: 25–36, 2008.
[6] Stephen G Walker et al. *J Bacteriol*, **176**: 6312–6323, 1994.
[7] P. Awram and J. Smit. *Microbiology*, **147**: 1451–60, 2001.
[8] N. Ravenscroft et al. *J Bacteriol*, **174**: 7595–605, 1992.
[9] N. Ravenscroft et al. *J Bacteriol*, **173**: 5677–84, 1991.

carbohydrate structures in *C. crescentus* cell envelope have now been solved.

2.2 Results

2.2.1 Initial assessment and component analysis

The polysaccharide (PS) was released from the LPS by hydrolysis with acetic acid. ^{1}H nuclear magnetic resonance spectroscopy (NMR) spectrum of the PS (fig. 2.1)) contained a large number of partially overlapping signals of various intensities in the anomeric region. It was obviously not a regular polymer with well-defined repeating units. Attempts to separate this material by anion-exchange chromatography led to the isolation of a number of fractions from neutral to slightly retained, but all of them had virtually identical NMR spectra. Methylation of the polysaccharide led to the identification of 3- and 3,4-substituted mannopyranose, terminal glucopyranose (derived from side-chain 3-O-methyl-glucose), terminal, 3-, 4-, and 2,4-substituted rhamnopyranose, 3-substituted Rha4NAc, and an unidentified derivative resembling methylated Rha4N that eluted between dimethylhexose derivatives and 3-substituted Rha4NAc. To identify the position of the methyl groups in naturally methylated monosaccharides, methylation was conducted with CD_3I. This confirmed the identification of tetramethylglucitol as originating from 3-O-methyl-glucose, but did not identify any other naturally methylated monosaccharides, visible in NMR spectra. An unknown derivative received two deuterated methyl groups.

2.2.2 O-Antigen structure determination (PS1)

A set of 2D spectra [gradient correlation spectroscopy (GCOSY), total correlation spectroscopy (TOCSY), nuclear Overhauser effect spectroscopy (NOESY), ^{1}H-^{13}C gradient heteronuclear single quantum coherence (GHSQC), gradient heteronuclear multiple bond coherence (GHMBC)] was obtained for the PS. There were many (more than 20) lines of correlations from the anomeric signals. Later, after the analysis of PS degradation products, most of them could be assigned to particular structures (figs. 2.2, 2.4, and 2.5). Polysaccharide heterogeneity was not caused by random acetylation, but PS contained 4 methyl groups (one major and 3 minor). Monosaccharide analysis revealed L-Rha, D-Man, D-Rha4N (perosamine), and 3-O-methyl-D-glucose. Other methylated monosaccharides were not identified by GC-MS as alditol acetates, possibly due to low content or degradation during hydrolysis.

In an attempt to simplify the structure, PS was oxidized with $NaIO_4$, reduced with $NaBD_4$, hydrolyzed with 2% AcOH, and the products were separated on a Biogel P6 column to give a polymer and an oligosaccharide (OS), OS1. Analysis of OS1 will be described below. For some reason not all of the rhamnan was oxidized, and some of its signals persisted in the spectra of the remaining polymer (without side-chain Rha F). To remove the rest of it, the oxidation was repeated to produce PS1. Spectra still contained some signals of minor components, analyzed later. Assignment of the spectra of the non-oxidizable polymer PS1 was difficult due to complete or partial overlap of the H-2,3,4,5 signals of Rha4NAc. To improve signal spread, PS1 was deacylated with 4 M NaOH. At this point the major polymer became positively charged

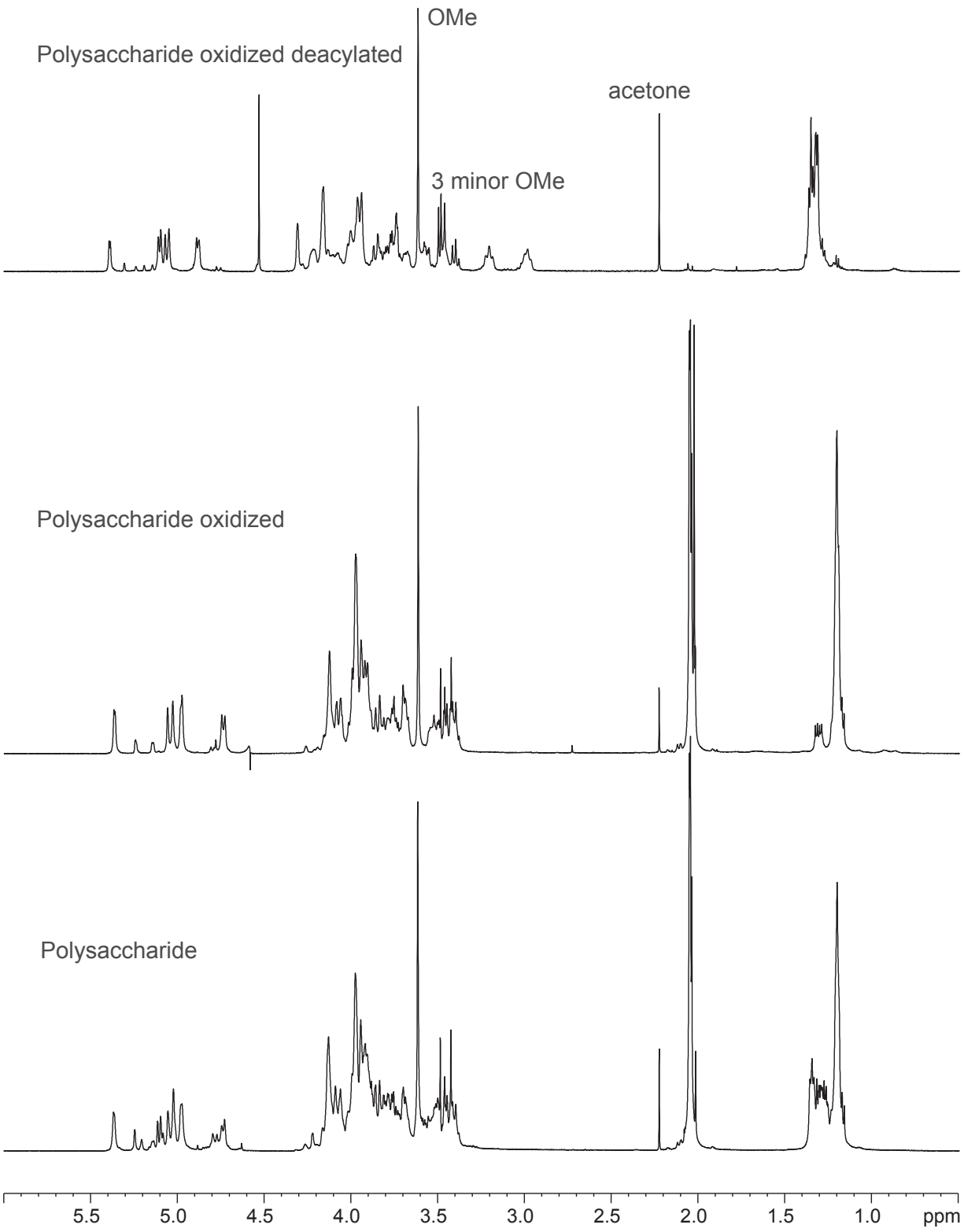
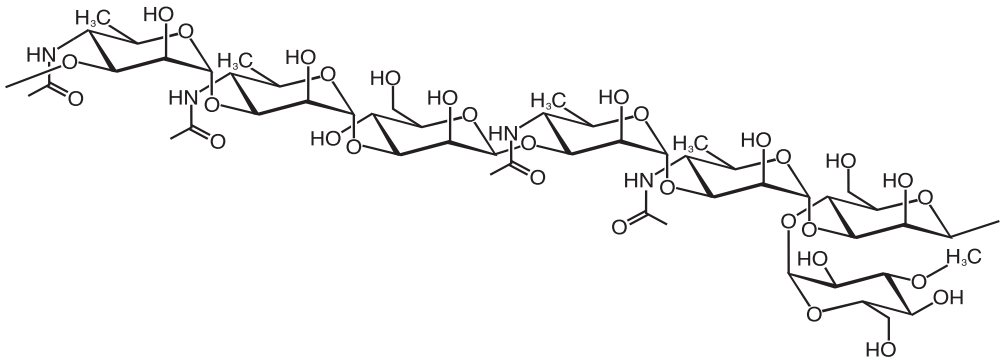


Figure 2.1: ^{1}H NMR spectra of the intact *C. crescentus* OPS (bottom trace), double oxidized polysaccharide (middle trace) and N-deacylated double oxidized polysaccharide (upper trace).

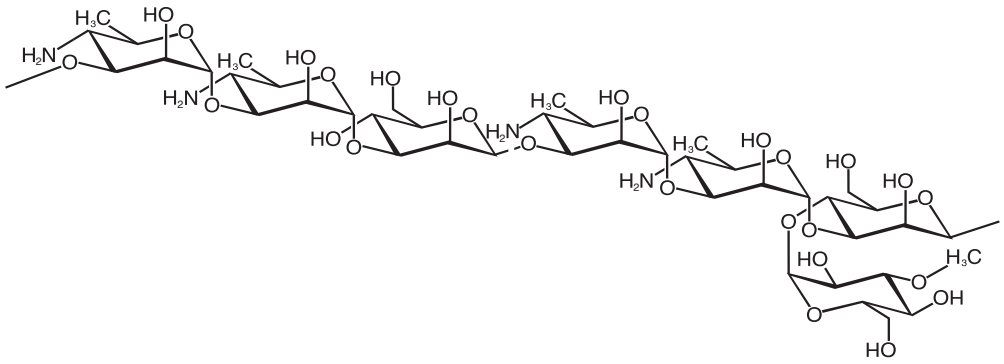
and an attempt was made to separate it from the minor components using cation-exchange chromatography. However, all material was eluted together at high salt concentration, thus indicating that all components were chemically bound together. Assignment of the spectra (fig. 2.3, table 2.1) became possible at this stage due to better signal spread (H-4 signals of Rha4N moved to high field due to deacylation) and the sequence shown on fig. 2.2 was proposed. Spectra contained the signals of two β-mannopyranose, 3-O-Me-α-Glcp, and two -Rhap4N. The following interresidual nuclear Overhauser effect (NOE) and heteronuclear multiple bond coherence (HMBC) correlations were used to determine the sequence: R1:L3, L1:Z3, Z1:Q3, Q1:W3, W1:X3, A1:X4. PS1 had trisaccharide repeating units composed of β-mannose and two α-Rha4NAc residues, and every second repeating unit carried a side branch of 3-O-Me-Glc. It seems that side-chains were present quite regularly at each second trisaccharide repeat of the main chain, because NOE correlations were observed between the repeating units with and without 3-OMe-Glc, and not between units of the same structure. Thus altogether, the repeating unit contained seven monosaccharides.

A



-3-*a*-PerNAc*p*-3-*a*-PerNAc*p*-3-*β*-Man*p*-3-*a*-PerNAc*p*-3-*a*-PerNAc*p*-3-*β*-Man*p*-
|4
a-Glc3Me*p*

B



-3-*a*-PerN*p*-3-*a*-PerN*p*-3-*β*-Man*p*-3-*a*-PerN*p*-3-*a*-PerN*p*-3-*β*-Man*p*-
|4
a-Glc3Me*p*

Figure 2.2: The structure of the *C. crescentus* OPS. **A.** The intact repeating unit, PS1. **B.** The deacylated product of PS1.

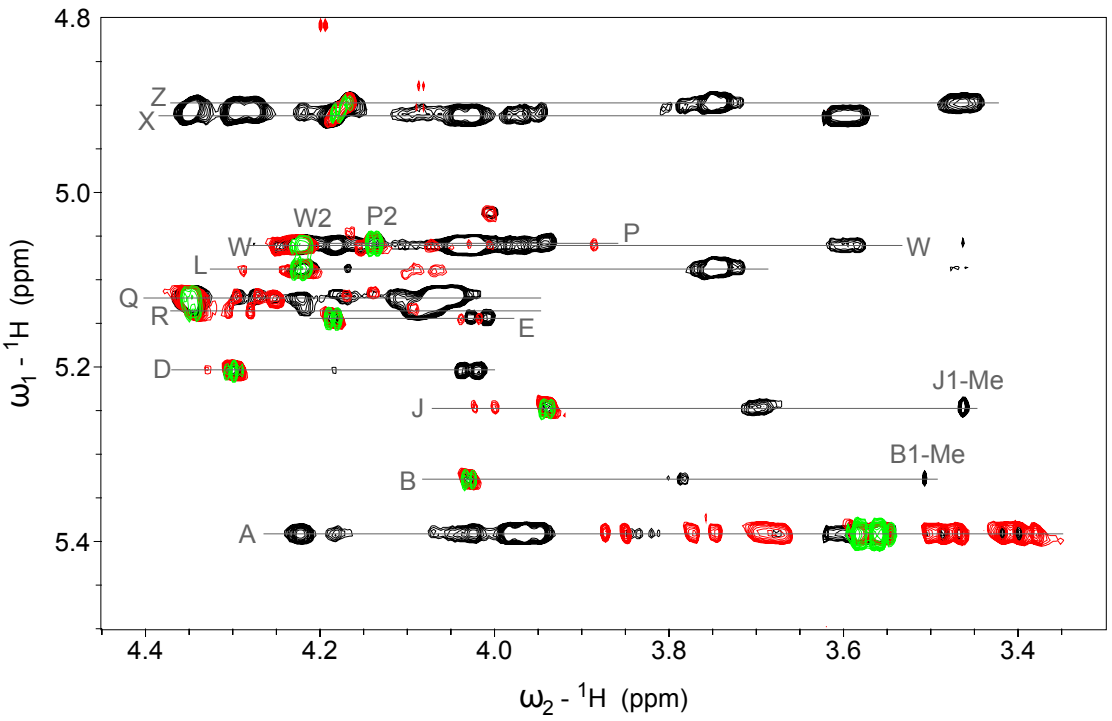
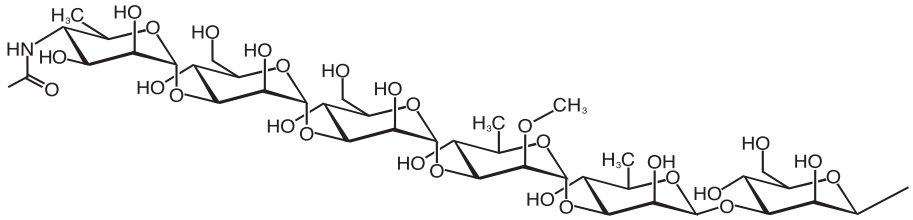


Figure 2.3: Overlap of COSY (green), TOCSY (red) and ROESY (black) correlations from anomeric protons of double oxidized deacylated *C. crescentus* polysaccharide PS1.

2.2.3 Minor component determination

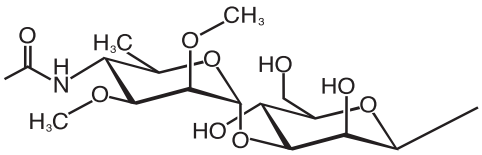
PS and PS1 spectra contained signals of minor components, which could not be removed by chromatography, as described above. They probably represented the non-reducing ends of the major chain, PS1 (fig. 2.4). The minor components contained methylated Rha (2-O-Me-Rha residue J and 2,3-Me2-Rha4N residue B). The position of the methyl groups were found from HMBC correlations between protons of methyl groups and carbon atoms bearing OMe groups, which all were well visible and did not overlap with other signals due to their low field position. Thus, two independent structural fragments, 1 and 2, were found and are shown in fig. 2.4. Mannose residues Z' and Z" at the non-reducing ends of these fragments were further linked to Rha4N residues, indistinguishable from the Rha4N of the main chain. Rha4N residue D had upfield shifted C-2 and downfield shifted C-3 signals (table 2.2)), which have not been explained. It appears that its O-3 was phosphorylated, producing typical phosphorylation signal shifts and broadening of the H-3 signal, but ^{1}H-^{31}P heteronuclear multiple-quantum correlation spectroscopy (HMQC) NMR spectrum showed no signals, possibly due to the low abundance of this residue. Possibly Rha residues inserted in the structure resembling PS1 represented the attachment point of the rhamnan (PS2) to PS1, if they were linked together.

A



a-Rha4NAc*p*-3-*a*-Man*p*-3-*a*-Man*p*-3-*a*-Rha2Me*p*-3-*β*-Rha*p*-3-*β*-Man*p*-PS

B



a-Rha4NAc2,3Me*p*-3-*β*-Man*p*-PS

Figure 2.4: The structure of minor component, the end caps of the OPS. **A.** Fragment 1. **B.** Fragment 2.

2.2.4 Rhamnan polysaccharide determination (PS2)

Periodate oxidation of the PS produced an OS1, which was analyzed by NMR and its structure, as shown on fig. 2.5, was determined using standard 2D NMR methods. Signal assignment is shown in the table 2.3. It contained three rhamnopyranose units and 4-deoxy-1-deutero-erythritol, produced by the oxidation-reduction of 4-substituted rhamnose. Formation of this oligosaccharide could be explained by oxidation of the side chain Rha F and 4-substituted Rha G in the PS1 (letter labels for monosaccharides were given using anomeric signals in the whole PS spectra starting from low-field). The unoxidized 4-substituted residue T in the oligosaccharide originally carried side-chain Rha F at position 2. Knowing the OS1 structure the signals of a corresponding polymer (PS2) were identified in the spectra of the whole PS, and are given in the table 2.3.

2.2.5 Core oligosaccharide determination

The core oligosaccharide of the *C. crescentus* LPS isolated after AcOH hydrolysis contained one non-degraded Kdo, two LD-Hep, one DD-Hep, mannose, galactose, and glucuronic acid in pyranose form. 2D NMR analysis led to the structure shown on fig. 2.6 (NMR assignments are in table 2.4, the heteronuclear single quantum coherence (HSQC) spectrum is in fig. 2.7). The sequence followed from the observed NOE: E1:C5,C7,F5; F1:E2; G1:F3; H1:C7,E2; K1:C4; L1:K4. Correlation E1:C7 is always observed in the α-Hep-5-Kdo fragment. E1:F5 was due to the α-Man-2-Hep linkage. H1E2 indicates spatial

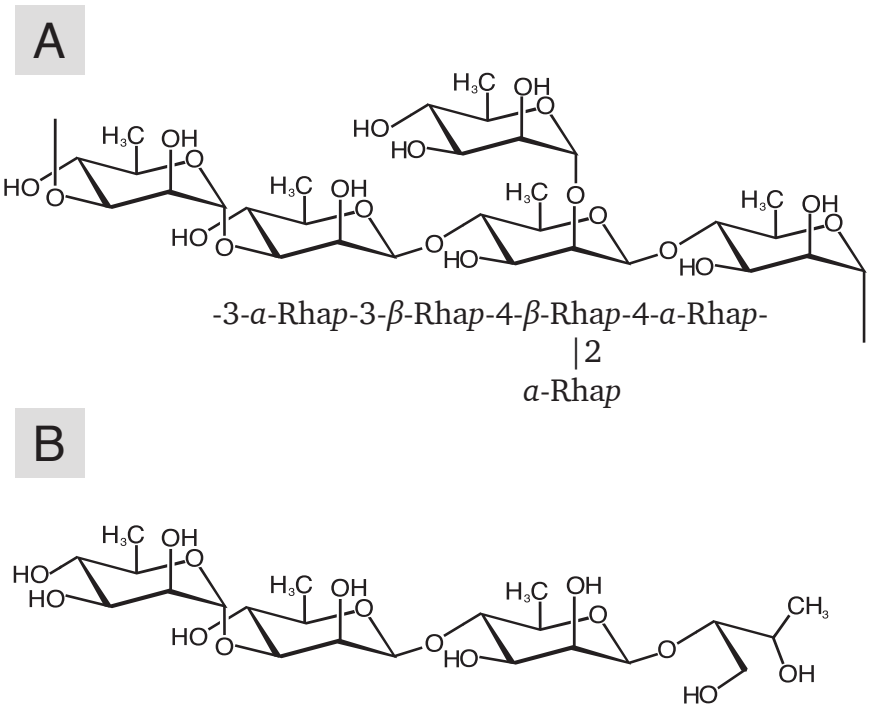


Figure 2.5: The structure of the *C. crescentus* rhamnan. **A.** the intact rhamnan, PS2. **B.** the oxidised rhamnan product, OS1.

proximity of the residues E and H, linked to the same Kdo C. All expected transglycoside correlations were observed in HMBC spectrum, together with intra-ring correlations H-1:C-3 and H-1:C-5 for all α-pyranoses. Methylation analysis revealed terminal DD-Hep, terminal and 2-substituted LD-Hep, 3-substituted Man and terminal Gal. The structure agreed with mass spectral data, electrospray ionization (ESI) negative $[M\text{-}H]^-$ = 1314.9, $[M\text{-}2H]/2^-$ = 656.7, calculated exact mass Hex2Hep3HexA1Kdo1 = 1314.4 Da.

2.3 Methods

2.3.1 Bacterial strain construction and growth conditions

The strain used for the preparation of LPS was JS1025, a derivative of *C. crescentus* CB15 NA1000. The salient features are that it has an engineered amber mutation in *rsaA* leading to the loss of the S-LAYER and the gene CCNA_00471 has been inactivated by a partial deletion. CCNA_00471 encodes a putative GDP-L-fucose synthase.[10] The knockout (Δ471) confers a deficiency in an EPS that was previously found to contain L-fucose.[9] CCNA_00471 was disrupted in the same manner as previously in JS4038,[11] except the

[10] M. E. Marks et al. *J Bacteriol*, **192**: 3678–88, 2010.
[11] C. Farr et al. *PLoS One*, **8**: e65965, 2013.

starting strain used here was JS1023.[12]

Cells were grown to mid-to-late log phase (OD_{600} = 0.9) in M16HIGG defined medium at 30°C in 2.8 l Fernbach flasks containing 1250 ml of medium, shaking at 100 rpm. M16HIGG is a modification of M6HIGG medium,[13] containing 0.31% glucose, 0.09% glutamate, 1.25 mm sodium phosphate, 3.1 mm imidazole, 0.05% ammonium chloride and 0.5% modified Hutner's Mineral Base.[14]

2.3.2 LPS isolation

LPS was isolated from the cells via disrupting the outer membrane by chelation. The protocol was a modification of the procedure reported by Walker *et al.* [6] Cells were centrifuged at 12 400 x g for 10 min. The pellets were suspended with distilled water and recentrifuged. These pellets were resuspended in 1/10 original culture volume in phosphate-buffered saline (PBS)[15] amended with 35 mm ethylenediaminetetraacetic acid (EDTA), agitated at room temperature for 10 min and then centrifuged at 15 300 x g for 15 min. The supernatant was retrieved and re-centrifuged, as before, to ensure clarity and then dialyzed against 5 mm $MgCl_2$. DNase and RNase were added to final concentrations of 10 $\mu g\,ml^{-1}$ and 100 $\mu g\,ml^{-1}$, respectively, and incubated at 37°C for 2 h. Proteinase K was added to a final concentration of 0.3 $mg\,ml^{-1}$ and the preparation was incubated at 50°C overnight. The sample was then ultracentrifuged at 184 000 x g for 3 h. Glassy pellets formed which were suspended in distilled water to 1/100 original culture volume. A Bligh-Dyer extraction was performed to reduce contaminating lipids.[16]

2.3.3 Bligh Dyer Extraction

A Bligh Dyer extraction was performed on all LPS preparations to reduce the presence of contaminating lipids. The extraction was performed as first published.[16] In short, to one volume of aqueous sample, 3.75 volumes of chloroform/methanol (1:2 v/v) was added and the sample was vortexed for 30 seconds. 1.25 volumes of chloroform was added and the sample was vortexed again for 30 seconds. 1.25 volumes of water were added and the sample was vortexed a final time for 30 seconds.

2.3.4 Gel electrophoresis

Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 13% separating gel.[17] Detection of LPS was done by periodate oxidation and silver staining as described by Zhu *et al.* [18]

[12] F. Amat et al. *J Bacteriol*, **192**: 5855–65, 2010.

[13] J Smit, Mark Hermodson, and N Agabian. *J Biol Chem*, **256**: 3092–3097, 1981.

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[17] Ulrich K Laemmli. *Nature*, **227**: 680–685, 1970.

[18] Zhong-Xin Zhu et al. *Electrophoresis*, **33**: 1220–1223, 2012.

2.3.5 NMR spectroscopy

NMR experiments were carried out on a Varian INOVA 600 MHz (1H) spectrometer with 5 mm gradient probe at 25–50°C with acetone internal reference (2.225 ppm for 1H and 31.45 ppm for 13C), using standard pulse sequences GCOSY, TOCSY(mixing time 120 ms), ROESY (mixing time 300 ms), GHSQC, and GHMBC(100 ms long range transfer delay), HMQC for ^{1}H-^{31}P correlation, JHX set to 10 Hz. AQ time was kept at 0.8–1 sec for H-H correlations and 0.25 sec for HSQC. 256 increments were acquired for t_1 in all 2D spectra, except 512 for GCOSY.

2.3.6 Chromatography

Gel chromatography was performed on a Sephadex G-15 column (1.5x60 cm) or a Bio-gel P6 column (2.5x60 cm) in pyridine-acetic acid buffer (4 ml:10 ml:1 l water), and monitored by refractive index detector (Gilson). Anion exchange chromatography was done on an Hitrap Q column (2x5 ml size, Amersham), with ultraviolet Light (UV) monitoring at 220 nm in a linear gradient of NaCl (0–1 M, 1 h) at the 3 ml min^{-1}. Fractions of 1 min were collected and additionally tested for carbohydrates, by spotting on an SiO_2 thin-layer chromatography (TLC) plate, dipping them in 5% H_2SO_4 in EtOH and heating with a heat-gun. All fractions of interest were dried in a Savant drying centrifuge and 1H spectra were recorded for each fraction without desalting. For 2D NMR, desalting was performed on a Sephadex G15 column.

2.3.7 Monosaccharide analysis

Samples with added inositol standard were hydrolyzed with 3 M trifluoroacetic acid (TFA) at 120°C. Monosaccharides were converted to alditol acetates by conventional methods and identified by gas chromatography-mass spectroscopy (GC-MS) on a Varian Saturn 2000 instrument on a DB17 capillary column (30 m x 0.25 mm ID x 0.25 µm film) with helium carrier gas, using a temperature gradient 170°C (3 min), 250°C at 5°C min^{-1}.

2.3.8 Determination of absolute configurations of monosaccharides

To the polysaccharide sample (0.2 mg) (R)-2-BuOH (0.2 ml) and acetyl chloride (0.02 ml) were added at room temperature, heated at 90°C for 2 h, dried by air stream, acetylated, analyzed by GC-MS as described above. Standards were prepared from monosaccharides of known configuration with (R)- and (S)-2-BuOH.

2.3.9 Methylation analysis

For the methylation analysis core sample (2 mg) was dephosphorylated with 50 µl of 48% HF for 20 h at +10°C, diluted with 2 ml of ethanol, precipitate collected by centrifugation, washed with 2 ml of ethanol, dried.

Methylation was performed by Ciucanu-Kerek procedure.[19] 0.5 mg of the sample was dissolved in 0.5 ml of dry DMSO with heating at 100°C for 5-10 min until complete dissolution. Powdered NaOH (about 50 mg) was added and the mixture was stirred for 30 min. 0.2 ml of MeI was added and the mixture was stirred for a subsequent 30 min. The sample was then flushed with air to remove the MeI and diluted to 10 ml with water. The sample was passed through a C18 Seppak cartridge, washed with 10 ml of water, and then the methylated compound was eluted with 5 ml of methanol. The methylated product was hydrolyzed with 3 M TFA (120°C, 3h), dried, reduced with $NaBD_4$, and the reagent destroyed with 0.5 ml of 4 M HCl. The solution was dried under a stream of air and dried twice more with the addition of MeOH (1 ml). The sample was acetylated with 0.4 mlAc_2O and 0.4 ml pyridine for 30 min at 100°C. It was then dried and analyzed by GC-MS.

2.3.10 Periodate oxidation

PS (10 mg) was dissolved in water (2 ml). $NaIO_4$ (20 mg) was added and the solution was incubated at room temperature for 24 h. Ethylene glycol (0.2 ml) and an excess $NaBD_4$ were added. The solution was then kept for 1 h before being treated with 0.2 ml of AcOH and desalted on a Sephadex G-15 column. The product was hydrolyzed with 2% AcOH, 2 h at 100°C, and separated on a Sephadex G-50 column to give OS1.

2.4 Discussion

The LPS of *C. crescentus* has an unusually complicated structure with two different polysaccharides, irregular substituents, and unfavourable NMR spectra. Presented data show structures of the core part, two polymers, and putative terminal structures. The polysaccharides could not be separated by size exclusion or anion-exchange chromatography and are probably linked together through the same core. The core of the *C. crescentus* LPS has been studied previously and an initial assessment of its composition was made;[8] but the complete structure had not been determined. The structure of the OPS has not been studied before. In our view the polysaccharide structure of the *C. crescentus* LPS represents one of the most complicated bacterial LPS polysaccharide structures identified so far.

The Kdo present in the LPS core structure (fig. 2.6) has the typical substitutions at O-4 and O-5 of a manno-configured sugar and a negatively charged sugar, respectively.[20] It also has a rarely observed third substitution at O-7 with a heptose moiety. The Kdo O-7 position is known to be occupied by a galactose moiety in the core of *Rhizobium leguminosarum* bv. Viciae VF39;[21] and the secondary Kdo in the core oligosaccharide from *Acinetobacter baumannii* ATCC 19606 has an O-7 substituted with a glucosamine.[22]

In the traditional model LPS occupies the outer leaflet of the outer membrane of a Gram-negative bacterium, and so (excepting the presence of cell associated EPS) is the outermost layer of the cell. For

[19] Ionel Ciucanu and Francisc Kerek. *Carbohyd Res*, **131**: 209–217, 1984.
[20] Helmut Brade. *Endotoxin in health and disease*. New York: Marcel Dekker, 1999. xviii, 950 p.
[21] Y. Zhang, R. I. Hollingsworth, and U. B. Priefer. *Carbohyd Res*, **231**: 261–271, 1992.
[22] E. V. Vinogradov et al. *Eur J Biochem*, **243**: 122–127, 1997.

C. crescentus, however, LPS is the penultimate barrier below the protein S-LAYER. The *C. crescentus* OPS serves as the anchor for the S-layer and is likely not accessible to the environment.[6] The carbohydrates found in the OPS are particularly hydrophobic, marked by the abundance of deoxy-sugars, acetyl groups, and methyl groups. This hydrophobicity is possibly a result of particular sugars needed for S-LAYER anchoring, as these carbohydrate structures likely evolved as the cognate ligands for the S-LAYER protein, RsaA. The distance between the S-LAYER and the outer membrane is about 17–19 nm.[23] It is possible the hydrophobicity aids in packing the polysaccharides between the S-layer and the LPS. Further determination of RsaA's structure should help illuminate the interaction between the S-layer and OPS.

Knowledge of the structure of *C. crescentus* OPS and LPS will facilitate the determination and characterization of their biosynthetic enzymes and mutant variants. Already, the enzymes LpxI[24] and GDP-L-perosamine acetylase[25] from *C. crescentus* have been characterized. One uncharacterized enzyme, WbqL, is necessary for proper OPS synthesis and disruption of wbqL leads to the accumulation of truncated and S-layer anchoring deficient OPS in the inner membrane and inhibits Crescentin-mediated cell curvature.[26] Many genes, such as wbqL, have been identified as essential for OPS synthesis[7] but have not yet been characterized. Other genes, that must be essential for OPS synthesis, have yet to be identified or characterized, such as the O-antigen polymerase and ligase.

The subunit-based repeating nature of *C. crescentus* OPS suggests that a Wzy-dependent pathway synthesizes the polymer.[27] The previous study that aimed to identify genes essential for OPS did not identify many of the canonical genes in the Wzy-dependant pathway,[7] such as the O-unit transporter, Wzx, O-antigen polymerase, Wzy; the chain-length determinate protein, Wzz; and the O-antigen ligase, WaaL. Genes that have been annotated as putative O-antigen synthesis genes do appear in the sequenced genomes for *C. crescentus* CB15, but they have not been experimentally confirmed.

An additional aspect to this LPS it the fact that its O-antigen is of homogenous length. While other LPSs vary in size due to the number of O-antigen repeat groups, appearing as a laddering of bands by SDS-PAGE, the LPS from C. crescentus appears as a single band.[6] Initial matrix assisted laser desorption/ionization-time of flight mass spectroscopy (MALDI-TOF) analysis of the entire LPS indicates a size of about 10.8 kDa (not shown). After accounting for the solved structures for the lipid A and core regions, this suggests the LPS contains approximately 5 repeats of the proposed heptameric O-antigen structure. There is not currently a known mechanism for the regulation and synthesis of a strictly homogenous length O-antigen. It is possible that this OPS is synthesized via the ATP-binding cassette (ABC)-transporter-dependent pathway[27] or another heretofore undiscovered mechanism. In any event it would seem that the transfer of a polysaccharide of this considerable size to the outer leaflet of the outer membrane is a remarkable feat for the bacterium.

[23] A. Moll et al. *Mol Microbiol*, **77**: 90–107, 2010.
[24] Louis E Metzger IV et al. *Nat Struct Mol Biol*, **19**: 1132–1138, 2012.
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Table 2.1: NMR data for *C. crescentus* PS1 (40°C) and deacylated PS1 (50°C). Me at 3.62/61.3 ppm.

PS1							
		1	2	3	4	5	6
Rha4NAc R	H	4.97	3.98	4.13	3.92	3.91	1.21
	C	103.3	68.2	75.5	52.4	69.4	18.0
Rha4NAc Q	H	4.98	3.97	4.11	3.92	3.91	1.21
	C	103.3	68.2	75.5	52.4	69.4	18.0
Rha4NAc L	H	5.05	4.12	3.99	3.98	3.98	1.21
	C	103.3	70.4	78.2	53.0	69.4	18.0
Rha4NAc W	H	5.02	4.12	3.96	3.98	3.98	1.21
	C	104.0	70.4	77.8	53.0	69.4	18.0
β-Man X	H	4.74	4.09	3.98	3.90	3.54	3.80; 3.96
	C	97.8	72.1	85.4	72.1	75.8	62.6
β-Man Z	H	4.72	4.06	3.70	3.68	3.41	3.77; 3.96
	C	98.2	71.9	82.3	67.2	77.3	62.4
Glc3Me A	H	5.36	3.51	3.40	3.44	3.68	3.75; 3.85
	C	100.0	72.2	84.1	70.1	74.1	61.6
Deacylated PS1							
Rha4N R	H	5.13	4.34	4.29	3.30	4.18	1.38
	C	103.5	67.4	75.0	53.4	67.8	18.0
Rha4N Q	H	5.11	4.34	4.29	3.30	4.18	1.38
	C	103.5	67.4	75.0	53.4	67.8	18.0
Rha4N L	H	5.08	4.22	4.06	3.11	4.09	1.35
	C	103.9	69.9	79.9	53.6	69.5	18.0
Rha4N W	H	5.06	4.22	4.06	3.11	4.09	1.35
	C	103.9	69.9	79.9	53.6	69.5	18.0
β-Man X	H	4.91	4.18	4.03	3.96	3.59	3.83; 3.96
	C	98.0	71.7	85.0	71.7	75.9	62.1
β-Man Z	H	4.90	4.18	3.75	3.75	3.46	3.83; 3.96
	C	98.0	71.7	82.0	67.0	77.4	62.1
Glc3Me A	H	5.39	3.57	3.40	3.48	3.68	3.76; 3.86
	C	100.2	72.2	84.0	70.0	74.1	61.7

Table 2.2: NMR data for the minor components of the double oxidized non-deacylated PS (50°C). Methyl group signals: B2: 3.48/59.5; B3: 3.42/57.9; J2: 3.45/59.6 ppm (H/C)

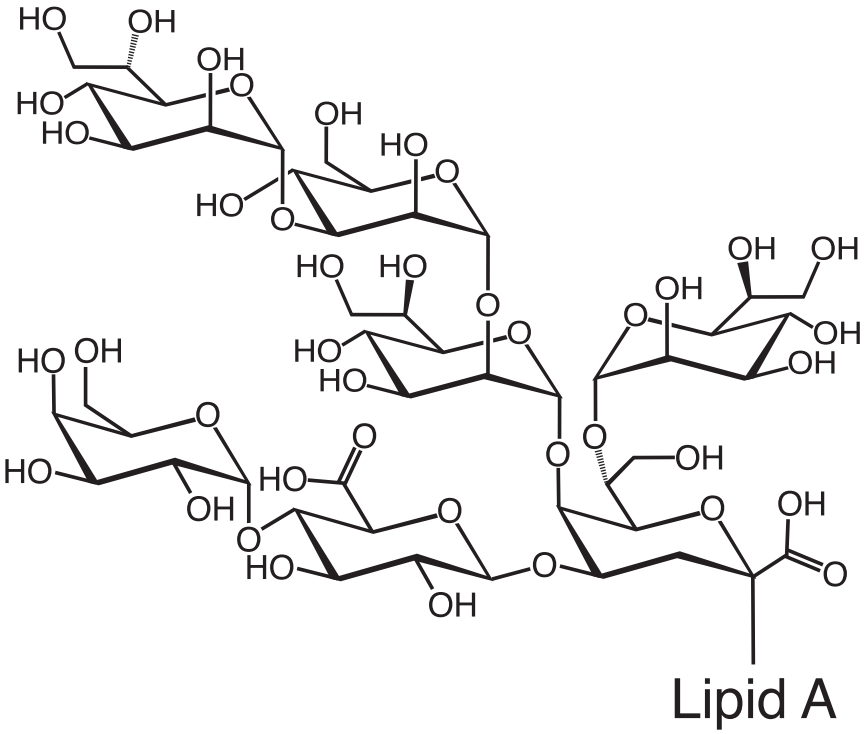
		1	2	3	4	5	6
Rha4N2,3Me B	H	5.25	3.96	3.71	3.81	3.91	1.17
	C	100.2	76.2	78.2	53.2	69.5	18.0
α-Rha2Me J	H	5.25	3.95	4.02	3.45	3.90	1.30
	C	100.2	75.9	75.4	71.8	69.5	18.0
α-Rha4N D	H	5.14	4.27	4.20	3.95	3.97	1.23
	C	100.1	67.4	75.2	52.2	69.4	18.0
α-Man E	H	5.13	4.17	3.99	3.75	3.84	
	C	100.1	70.9	79.8	67.1	74.4	
α-Man P	H	5.05	4.14	4.00	3.85	3.96	
	C	97.6	71.0	79.5	66.9	74.0	
β-Rha U	H	4.78	4.16	3.69	3.48	3.44	1.32
	C	100.9	71.8	82.0	72.4	73.1	18.0
β-Man Z"	H	4.72	4.06	3.75	3.75	3.46	3.83; 3.96
	C	98.2	71.9	86.0	67.0	77.4	62.1
β-Man Z'	H	4.72	4.06	3.73	3.75	3.46	3.83; 3.96
	C	98.2	71.9	82.3	67.0	77.4	62.1

Table 2.3: NMR data for *C. crescentus* PS2 and its $NaIO_4$ oxidation product OS1 (40°C).

		1	2	3	4	5	6
α-Rha N, OS1	H	5.04	4.07	3.85	3.47	3.85	1.30
	C	103.1	71.1	71.1	72.9	69.9	17.6
α-Rha N, PS	H	5.02	4.02	3.79	3.45	3.72	1.28
	C	103.0	71.5	78.8	73.3	70.4	17.5
β-Rha T, OS1	H	4.78	4.13	3.71	3.54	3.53	1.33
	C	100.7	71.1	72.3	83.4	71.7	17.5
β-Rha T, PS	H	4.79	4.22	3.82	3.62	3.61	1.36
	C	101.7	76.7	73.5	83.4	73.2	17.5
β-Rha V, OS1	H	4.75	4.12	3.67	3.49	3.49	1.34
	C	101.1	71.4	81.3	71.9	72.9	17.5
β-Rha V, PS	H	4.77	4.13	3.66	3.66	3.50	1.35
	C	101.4	71.2	81.7	67.2	73.3	17.5
X (ox. G), OS1	H	3.69; 3.71	3.75	3.99	1.20		
	C	61.8	84.8	67.7	18.0		
α-Rha G, PS	H	5.10	4.13	3.94	3.58	3.90	1.35
	C	103.1	71.5	70.3	84.5		17.5
α-Rha F, PS	H	5.11	4.09	3.87	3.45	4.07	1.27
	C	102.4	72.1	71.3	73.3		17.5

Table 2.4: NMR data for the core oligosaccharide (25°C).

		1	2	3	4	5	6	7	8
Kdo C	H			2.05; 2.35	4.23	4.28	4.09	3.91	3.81; 3.93
	C		98.2	34.7	74.9	74.2	71.0	79.2	61.5
DLHep E	H	5.24	3.85	4.12	3.89	3.88	4.04	3.73; 3.73	
	C	101.1	80.2	71.1	67.3	74.1	70.0	63.9	
Man F	H	5.03	4.30	4.00	3.79	3.80	3.80; 3.91		
	C	103.7	70.6	78.1	67.5	74.5	62.0		
DDHep G	H	5.15	4.05	3.86	3.77	3.84	4.06	3.76; 3.84	
	C	103.1	71.0	71.8	68.7	74.5	72.9	62.9	
DLHep H	H	5.07	4.14	3.89	3.89	3.80	4.10	3.79; 3.81	
	C	102.7	73.7	71.8	67.3	73.2	70.2	64.9	
GlcA K	H	5.22	3.66	4.00	3.99	4.15			
	C	98.8	72.4	74.9	73.6				
Gal L	H	5.49	3.80	3.85	3.99	3.94	3.69; 3.69		
	C	99.9	69.7	70.3	70.0	71.8	61.6		



a-DLHep*p*
|7
a-DDHep*p*-3-*a*-Man*p*-2-*a*-DLHep*p*-5-*a*-Kdo*p*-
|4
a-Gal*p*-4-*a*-GlcA*p*

Figure 2.6: The structure of the *C. crescentus* core OS.

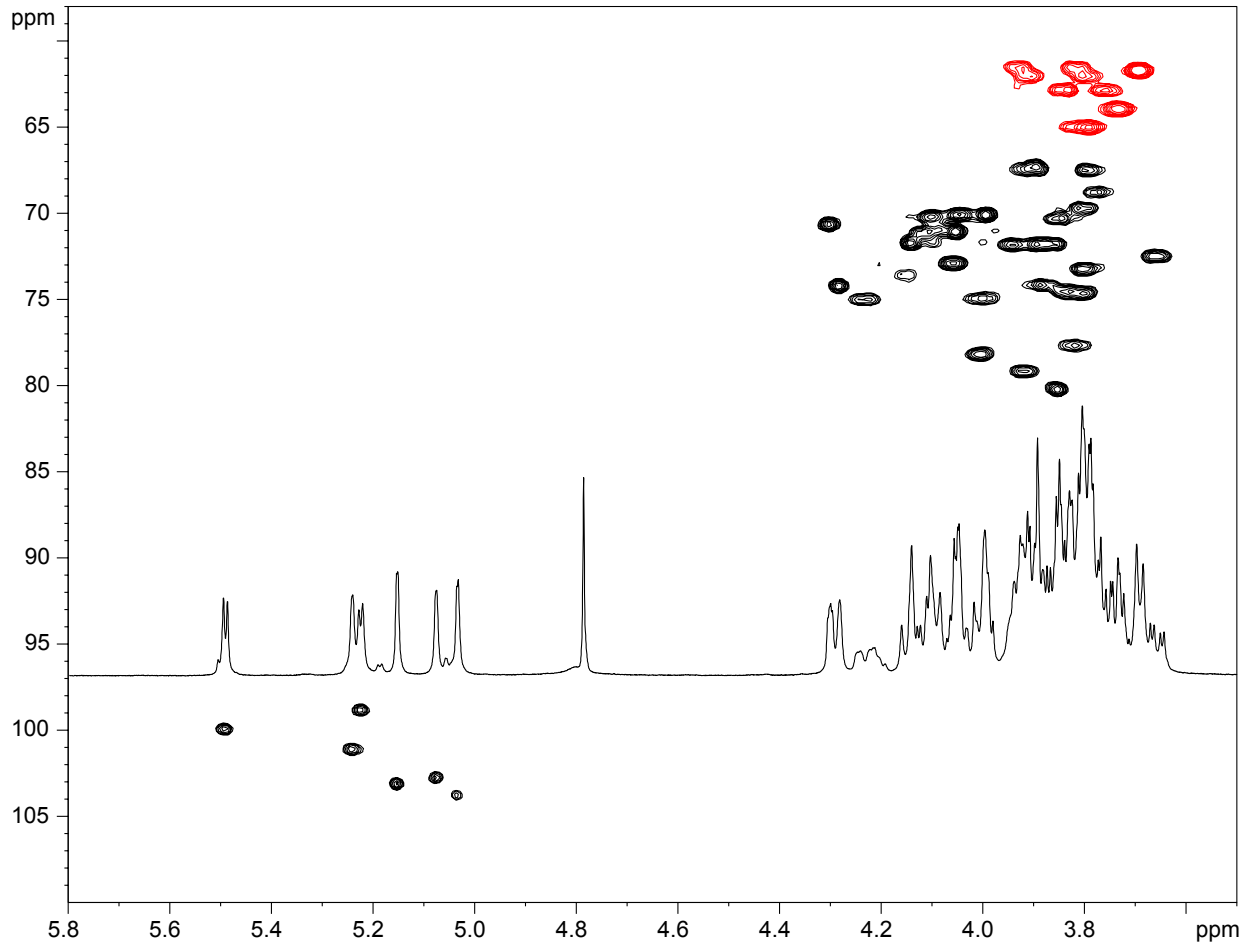


Figure 2.7: Fragment of ^{1}H-^{13}C HSQC spectrum of the core.

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Appendix A

Supporting Materials

This would be any supporting material not central to the dissertation. For example:

- Authorizations from Research Ethics Boards for the various experiments conducted during the course of research.
- Copies of questionnaires and survey instruments.