EVALUATION OF PULLULANASE FOR SECRETION
OF ENZYMATICALLY ACTIVE HETEROLOGOUS CELLULASES
AS CHIMERIC PROTEINS FROM
KLEBSIELLA OXYTOCA

by

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ABSTRACT

The objective of this research was to evaluate the potential of using the *Klebsiella oxytoca* secreted pullulanase (PulA) as a determinant for secretion of *Cellulomonas fimi* cellulases as fusion proteins. Fusion proteins were created between PulA from *K. oxytoca* and each of the following *C. fimi* cellulose hydrolyzing enzymes: endo-β-1,4-glucanase A (CenA), endo-β-1,4-glucanase B (CenB), and exo-β-1,4-glucanase (Cex). The fusion proteins PulA:CenA, PulA:CenB, and PulA:Cex were produced and found to retain cellulolytic activity when expressed in *Escherichia coli* cells. Because cellulose hydrolysis by CenB presented the greatest applied potential for degrading cellulose to cellobiose at the outset of my thesis research, PulA:CenB was studied in more detail. Several plasmid systems were tested for expression of the *pulA:cenB* fusion gene in *K. oxytoca* and the expression vector pMMB207, which allows for IPTG-regulated expression from the *tac* promoter, was determined to be the most appropriate. Expression of *pulA:cenB* led to the accumulation of 17 mg/L of the chimeric PulA:CenB within cultures of *K. oxytoca* (pMMBpulA:cenB). A portion (8%) of the total chimeric protein produced was located within the membrane-enriched fraction and shown to be exposed to the extracellular medium by immunolocalization, with ca. 6 - 16% of cell-associated CMCase activity located at the cell surface. This amount of cell-surface exposed PulA:CenB was not sufficient to allow growth on cellulose. Mutagenesis of *K. oxytoca* (pMMBpulA:cenB) cells with MNNG, followed by selection for growth on cellulose as sole carbon source was done, but mutants able to grow on cellulose were not isolated. Additionally, expression of PulA:CenB in *K. oxytoca* (pMMBpulA:cenB) cultures grown in a minimal medium resulted in a triphasic growth pattern in this strain, suggesting an abnormality in PulA:CenB membrane translocation that interfered with cell growth.
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1.0 INTRODUCTION

1.1 Background.

Cellulose, a large insoluble macromolecule composed of glucose joined in β-1,4-linkages, is one of the major structural components of the cell wall of plants as well as one of the most abundant natural polymers, and is a principle component of industrial and municipal waste (Figure 1; Kuhad et al. 1997). Individual cellulose molecules (containing from 100 to 25 000 polymerized glucose molecules) associate to form a microfibril stabilized through inter- and intra-molecular hydrogen bonds. Bundles of microfibrils are packed into fibrils which then come together to form cellulose fibres. Within cellulose fibres, the glucose molecules are organized in such a manner that a crystalline X-ray diffraction pattern can be obtained (Béguin and Aubert 1994). Therefore, although simple in chemical composition, the overall crystalline nature of the cellulose structure makes cellulose very stable and recalcitrant to degradation by both mechanical and biological processes (Soltes 1983; Callihan and Clemmer 1979).

Since the "energy crisis" of the mid 1970's, many groups around the world have attempted to develop technologies to release the energy (i.e., glucose) locked within the cellulose molecule for applied uses such as the production of single cell protein (Callihan and Clemmer 1979), chemicals (citric acid and glutamic acid among others; Knapp 1985), and ethanol (Wood and Ingram 1992). However, these technologies have been fraught with many difficulties stemming from the recalcitrant nature of the cellulose substrate to disruption and therefore, have had little commercial success. The processes that have been developed are energy intensive (to disrupt the cellulose fibre) and thus
Figure 1: Organization of glucose molecules within one cellulose microfibril. Hexagons represent glucose molecules attached in β-1,4-linkages within a cellulose microfibril.
rely on the coupling of cheaply available cellulose (usually agricultural waste material) and energy to be economically viable. However, as the cost of energy increases, these processes would likely become even less economically practical.

In British Columbia, two of the major sources of waste cellulose (1 - 3 tonnes/day) are by-products of the logging (sawdust) and pulp and paper (clarifier fines) industries (McCubbin 1983). The wood industry considers clarifier fines and sawdust to be waste materials, which are either burned on site as fuel or shipped to landfills for disposal, and consequently represent a significant loss of a renewable source of glucose. In principle, given correct biological conversion conditions, waste cellulose material could be converted into a commercially viable product as part of the downstream processing of waste water from a pulp and paper plant. One such product could be a biological fertilizer that when spread onto areas clear-cut by the logging industry might promote the growth of newly planted saplings, or which could be used in the horticultural industry.

Two changes in the nature of the cellulose molecule must occur prior to its use as a fertilizer. Firstly, the cellulose fibres must be disrupted and hydrolyzed to liberate the polymerized glucose subunits. As mentioned above, the cellulose fibre is very stable and thus high temperatures and acid or base treatments are required to hydrolyze and solubilize the substrate. Alternatively, microorganisms are known to hydrolyse cellulose and use the solubilized sugars as a carbon source for growth. Research conducted over the past 30 years has increased our understanding of the mechanisms employed by microorganisms to degrade cellulose. Therefore, it appears possible that a microorganism could be exploited to disrupt the cellulose fibre at ambient temperatures
without pretreatment with acid or base. Perhaps in this manner, the economics of cellulose conversion into useful products would be more favourable.

Secondly, the nitrogen content ($\text{NO}_3^{-}$ and/or $\text{NH}_4^+$) of the cellulose material would need to be greatly increased for its use as a plant fertilizer. Exogenously manufactured $\text{(NH}_4\text{)}_2\text{SO}_4$ could be added to cellulose such that it could be used as a fertilizer (150 lbs $\text{(NH}_4\text{)}_2\text{SO}_4$/ton of sawdust; Guernsey 1949). However the Haber-Bosch process to convert $\text{N}_2$ to $\text{NH}_3$ requires 200 atmospheres of pressure and $800^\circ \text{C}$ (White 1995). In this respect, the use of microorganisms which can convert $\text{N}_2$ to $\text{NH}_3$ at 1 atmosphere of pressure and ambient temperatures would be economical. The resulting biomass containing hydrolyzed cellulose, fixed nitrogen, and the bacteria responsible for this conversion could be used as a soil additive, which could provide a net increase in the total nitrogen content of soil for the stimulation of plant growth. Additionally, a waste product would be converted into a useful and commercially valuable product.

1.2 Biological nitrogen enrichment of cellulose.

A nitrogen fixing bacterium could be used to enhance the nitrogen content of cellulose. However, the nitrogen fixer itself requires a carbon source for growth. Therefore, the nitrogen content of cellulose could be increased biologically in one of two ways: 1) by establishing a co-culture between a nitrogen-fixing and a cellulose hydrolyzing species and 2) using strains of bacteria that both degrade cellulose and fix nitrogen. In the first instance the cellulolytic organism would hydrolyze cellulose, producing soluble glucodextrins (e.g. cellobiose) and glucose that could be used for growth by itself as well as by the nitrogen-fixing bacterium. A mixed culture of *Cellulomonas gelida* (a cellulose
degrading bacterium) and *Azospirillum* species or *Bacillus macerans* (both nitrogen fixing bacteria) able to grow on nitrogen-limited cellulose substrates was described (Halsall and Gibson 1985). Similarly, a co-culture of the cellulolytic fungus *Tricoderma harzianum* and the nitrogen fixing bacterium *Clostridium butyricum* was reported to degrade cellulose while fixing nitrogen under anaerobic conditions (Veal and Lynch 1984).

Alternatively, some bacteria are able to degrade cellulose and fix atmospheric nitrogen simultaneously. To date three bacterial species expressing both these properties have been isolated from nature: an unclassified Gram-negative bacterium from the bivalve *Psiloteredo healdi* (“shipworm” from marine waters; Waterbury et al. 1983) and two *Clostridium* sp.: *Clostridium* strain C7 (Leschine and Canale-Parola 1983) and *Clostridium cellulovorans* (Sleat et al. 1984).

These three organisms were all isolated from environments rich in cellulose but lacking fixed nitrogen (NO$_3^-$ and/or NH$_4^+$) and appeared to be promising candidates in cellulose bioremediation processes. However, these organisms exhibit stringent growth characteristics that would limit industrial use. The symbiont of *Psiloteredo healdi* requires 0.1-0.3 M NaCl for optimal growth and both *Clostridium* species are obligately anaerobic (Greene and Freer 1986; Leschine and Canale-Parola 1983; Sleat et al. 1984). Co-cultures similar to the ones described above could be used to degrade cellulose and enhance the nitrogen content of waste cellulose. The ideal partners of the co-culture would need to be located and found (or modified) to be compatible such that a stable association is formed. Therefore, these organisms have limited applicability in a standard pulp and paper plant bio-treatment lagoon where the NaCl concentration would be lower and the water would be oxygenated.
1.3 Nitrogen fixation.

Biological nitrogen fixation, conversion of the chemically unreactive $N_2$ to the biologically available $NH_3$ is a complex process that can be carried out by members of many different families of bacteria as well as some archaea. Although nitrogen fixation occurs under conditions of low oxygen tension or anaerobically, the microbes involved are aerobes, anaerobes, or facultative anaerobes. Furthermore, such microbes can be either symbionts with leguminous plants (eg. *Rhizobium* sp. in pea plants), non-leguminous plants (eg. *Frankia* sp. in alder tree roots), or free-living bacteria (eg. *Klebsiella* or *Azotobacter* sp.). Although both aerobes and anaerobes fix nitrogen, the nitrogenase enzyme complex must be protected from inactivation by oxygen. The aerobes that fix nitrogen may protect their enzymes by a high rate of respiration such that there is very little free oxygen in the cytoplasm, or by encapsulating the enzymes within a protective protein coat (White 1995).

1.3.1 Nitrogen fixation by *Klebsiella* species.

In *Klebsiella* species, the most extensively studied group of nitrogen fixing bacteria, nitrogen fixation requires 21 gene products which are part of a regulon organized in 6 adjacent operons and encode enzymes, electron transport proteins, and regulatory proteins (White 1995). Under favourable conditions (eg., low $NH_3$ concentration and low oxygen tension), a complex cascade is initiated resulting in the expression of NifH (known either as dinitrogenase reductase or Fe-protein component) and NifDK (known either as dinitrogenase or MoFe-protein component; Miller and Orme-Johnson 1992). NifH and NifDK function together (referred to as the nitrogenase enzyme complex) to catalyze the conversion of atmospheric $N_2$ to $NH_3$ as summarized in the equation:
\[ 16\text{ATP} + 8\text{H}^+ + \text{N}_2 + 8\text{e}^- \rightarrow 16\text{ADP} + 16\text{Pi} + 2\text{NH}_3 + \text{H}_2 \]

The successful transfer of *nif* genes from *K. pneumoniae* into the closely related *Escherichia coli* was reported (Dixon and Postgate 1972). The *E. coli* transconjugant (strain M7) stably maintained the *nif* genes and fixed nitrogen under anaerobic growth conditions. Therefore, the transfer of nitrogen fixation capability to cellulolytic bacteria is possible provided that appropriate genetic transfer and expression tools are available. Some of the proteins produced by the 6 operons must also be correctly inserted in the bacterial membrane in order to function as regulatory and transport proteins, and so analogous membrane translocation and insertion machinery must be present. Additionally, the bacterium’s metabolism would have to be appropriate, or modified, such that growth was possible under an oxygen tension suitable for nitrogenase (NifH/DK) activity. Therefore, although nitrogen fixing ability can be transferred to another bacterium, it would be simpler to modify a nitrogen fixing bacterium to express cellulase genes.

1.4 Biochemistry and genetics of cellulose degradation.

Although cellulose fibres are large crystalline and stable structures, several microorganisms produce enzymes that hydrolyse the glucose-glucose bonds of the cellulose molecule. These organisms each produce an array of secreted enzymes that hydrolyze either glucose bonds internal to the cellulose strand (endo-cellulolytic enzymes) or glucose bonds at the ends of the cellulose strand (exo-cellulolytic enzymes). The complete hydrolysis of cellulose (resulting in the glucose disaccharide cellobiose) requires both types of activity (the endoglucanase creates more open ends where the exoglucanase can attack). Once the soluble
glucodextrins are transported into the cell, they are further hydrolysed to glucose by the enzyme β-glucosidase (β-1,4-glucosidase; EC 3.2.1.21) before entry into general metabolic pathways (Wakarchuk et al. 1984).

The cellulolytic activity of the fungus *Tricoderma reesei* and the aerobic bacterium *C. fimi* have been extensively studied (Béguin 1990). *T. reesei* and *C. fimi* produce enzymes which once secreted can individually hydrolyze glucose bonds within the cellulose molecule. In contrast, *C. thermocellum* and other anaerobic cellulose degraders produce enzymes which once secreted associate into large multiprotein complexes known as cellulosomes (Béguin and Aubert 1994). Although the genetics of regulation have not been elucidated in all cases, expression of cellulases is inhibited by the presence of glucose, other easily metabolized substrates, or by the accumulation of the cellulose hydrolytic product cellobiose (Béguin and Aubert 1994).

1.4.1 *C. fimi* cellulases.

The Gram-positive, mesophilic bacterium *C. fimi* produces a variety of cellulose degrading enzymes including the well characterized endo-cellulolytic enzymes CenA and CenB (1,4-β-D-glucan glucanohydrolase; EC 3.2.1.4) and the exo-cellulolytic enzyme Cex (1,4-β-D-glucan cellobiohydrolase; EC 3.2.1.91; Gilkes et al. 1991; Figure 2). In addition to the above mentioned cellulases, the endoglucanase CenD (Meinke et al. 1993), the cellobiohydrolases CbhA and CbhB (Meinke et al. 1994; Shen et al. 1995), and the mixed function endo/exo-cellulase CenC (Tomme et al. 1996; Figure 2) have been recently identified.

CenA, CenB, and Cex cellulases are all modular enzymes (as are all cellulases studied to date) with catalytic domains separated from
Figure 2: Representation of the cellulases of *C. fim.* Open rectangles: CBD; BCBD: *Bacillus*-like CBD; Black rectangles: Pro-Thr-rich spacer regions; Stippled rectangles: catalytic domains; F3: fibronectin-like hinge region. Proteins are drawn to scale with respect to each other (molecular mass indicated below each cellulases name). Modified from Sandercock *et al.* (1996).
cellulose binding domains (CBD), which are thought to serve as anchors to cellulose for hydrolysis. CenA is produced with an amino-terminal CBD which is separated from a carboxy-terminal catalytic domain by a Pro-Thr-rich spacer region (Figure 2). Cex is synthesized with an amino-terminal catalytic domain separated from the carboxy-terminal CBD by a Pro-Thr spacer region (Figure 2). Similarly, CenB is synthesized with an amino-terminal catalytic domain separated from a carboxy-terminal cellulose binding domain (CBD) by alternating proline-threonine-rich and fibronectin-like spacer regions (Figure 2; Meinke et al. 1991). At the start of my research CenB was the only C. fimi enzyme known to hydrolyse cellulose to cellobiose (Meinke et al. 1991) and was thus singled out as a potential candidate for investigation. All C. fimi cellulases are secreted enzymes and are synthesized with amino-terminal signal peptides necessary for secretion.

Recently a reclassification of enzymes that degrade polysaccharides in cell-walls of plants, including CenA, CenB, and Cex, has been proposed (Henrissat et al. 1998). In this scheme, CenA and CenB are renamed Cel6A and Cel9A. Cex has been reclassified as a mixed function xylanase/exoglucanase called Xyn10A. The old classification of these enzymes is used in this thesis: CenA and CenB are referred to as endoglucanases and Cex as an exoglucanase.

1.4.2 Heterologous expression of C. fimi cellulases.

The genes encoding the known C. fimi cellulases have been cloned and attempts have been made to express them in heterologous hosts in order to exploit their potential for cellulose hydrolysis. C. fimi cellulases have been expressed in the Gram-positive Streptomyces lividans (MacLeod et al. 1993), the Gram-negative Rhodobacter
capsulatus (Johnson et al. 1988) and E. coli (Francisco et al. 1993; Owolabi et al. 1988; Guo et al. 1988; O’Neill et al. 1986) as well as the yeast Saccharomyces cerevisiae (Wong et al. 1988).

Expression of the C. fimi cex gene in S. lividans from a Streptococcus promoter resulted in the production of glycosylated Cex and the accumulation, in the culture supernatant, of 5.5 mg/L Cex after 40 hours of growth (MacLeod et al. 1993). The specific activity of the S. lividans expressed Cex protein was 11.2 U/mg, comparable to the specific activity of the C. fimi expressed protein. Similar to the situation where cex is expressed in C. fimi (and E. coli), when expressed in S. lividans a 42 kDa proteolytic product was detected. This 42 kDa product, corresponding to the Cex catalytic domain, was believed to be due to proteolysis within the Pro-Thr spacer region (Sandercock et al. 1996).

When C. fimi cenA, cex, and cenB were expressed in the Gram-negative R. capsulatus, very little of the recombinant proteins were secreted (Johnson et al. 1988). In this case, CenA was expressed as a fusion to the inner membrane protein PufB while Cex and CenB were expressed in their entirety (containing the wild type signal peptide required for secretion from C. fimi). However, these clones did not grow on medium with cellulose as sole carbon source, suggesting that insufficient amounts of cellulases were secreted.

In E. coli, expression of cenB or cex resulted in periplasmic localization of the corresponding protein (Owolabi et al. 1988; O’Neill et al. 1986). However, when cex was expressed as a fusion protein with the E. coli outer membrane OmpA (amino acids 46 - 159) and the lipoprotein signal peptide, approximately 90% of the total activity was anchored on the external side of the outer membrane.
(Francisco et al. 1993).

*S. cerevisiae* was engineered to express and secrete both CenA and Cex (Wong et al. 1988). Recombinant *S. cerevisiae* expressing CenA and Cex incubated with aspen poplar wood chips that had been pretreated with acid and supplemented with exogenous β-glucosidase resulted in the solubilization of 43% of the reducing sugars.

These studies indicate that *C. fimi* cellulase expression in heterologous hosts is possible, as is secretion into the culture medium. Expression and secretion was easily effected from the Gram-positive bacterium *S. lividans*. However, secretion in Gram-negative and eukaryotic yeast cells was only possible after significant alteration to include secretion signals from the respective heterologous host cell. Therefore, the possibility of *C. fimi* cellulase secretion from heterologous Gram-negative bacterial hosts exists if the appropriate secretion signals are present.

1.5 Experimental approach.

Given the limited success of finding natural isolates capable of nitrogen fixation and cellulose hydrolysis, an alternative approach to combine these functions would be by using recombinant DNA technologies. Because of the successes of cellulase secretion using heterologous secretion signals and the complex nature of nitrogen fixation (see Section 1.3), I thought that the most effective way to combine nitrogen fixation and cellulose hydrolysis was to choose an appropriate nitrogen fixing host for expression of subcloned cellulases.

*K. oxytoca* (formerly *K. pneumoniae*) is capable of nitrogen fixation (Mahl et al. 1965), intracellular accumulation and catabolism of
cellobiose (Brenner 1984), and secretion of the starch hydrolyzing enzyme pullulanase (PulA; EC 3.2.1.41; Michaelis et al. 1985), and so it was chosen as the system to test for combination of the biological processes of cellulose hydrolysis and nitrogen fixation specified by the same cell. Expression of the genes encoding cellulose hydrolytic enzymes in the nitrogen fixing host strain K. oxytoca, from an alternative promoter, would eliminate feedback inhibition by cellobiose of cellulase gene expression and would ensure that all the proteins required for nitrogen fixation are present. Furthermore, the PulA protein could potentially be used to create fusion proteins for secretion of cellulases (see Section 1.5.2).

1.5.1 Protein secretion from K. oxytoca.

PulA and all the PulA-specific secretion proteins are encoded by genes that are part of the maltose regulon originally described in E. coli (Schwartz 1987; d'Enfert et al. 1987). Transcription of all genes within the maltose regulon is induced by the positive activator protein MalT in the presence of maltose or maltotriose (Michaelis et al. 1985). Although both maltose and maltotriose are in vivo inducers of the maltose regulon, in vitro experiments indicated that the true inducer in E. coli is maltotriose (Raibaud and Richet 1987). Many maltose regulon promoters are strongly inhibited by glucose (catabolite repression), although the promoters of the PulA system appear to lack detectable binding sites for the cyclic AMP-binding protein and thus are not expected to be repressed by the presence of glucose (Schwartz 1987; Chapon and Raibaud 1985). PulA, encoded by the pulA gene, is transcribed divergently from the pulC-O operon (Figure 3). The pulC-O operon together with pulS encode 14 proteins essential for PulA secretion.
Figure 3: Chromosomal organization of the *pulC-O* and *pulAB* operons, and *pulS* of *K. oxytoca*. Horizontal arrows indicate transcriptional units and direction of transcription. MalT indicates the location of MalT activated promoters. Scale: 0.82 cm = 1 kb. Modified from Pugsley (1993).
Secretion of the 1071 amino acid (145 kDa) *K. oxytoca* PulA enzyme occurs via a two-step process (Figure 4; Kornacker and Pugsley 1989; Michaelis *et al.* 1985). In the first step, cytoplasmic PulA is exported to the periplasm using the Sec-dependent export pathway common to many Gram-negative bacteria (Pugsley *et al.* 1991). There is a requirement for the 19 amino acid PulA signal peptide (Kornacker and Pugsley 1989), the translocation chaperone SecB, SecA (and associated ATP hydrolysis), and the translocation channel formed in the inner membrane by SecD, E, F, and Y. The amino-terminal signal peptide, once in the inner membrane to secure translocation of the PulA protein, is cleaved by leader peptidase II (LspA), leaving a cysteine as the amino-terminal amino acid. Cleavage of the signal sequence by LspA is thought to occur simultaneously with modification of the amino-terminal cysteine by the addition of two fatty acyl groups in ester linkages to a glyceride residue, and the addition of a third fatty acyl group in direct amide linkage to the same cysteine (Pugsley *et al.* 1986). Once cleaved and modified, the lipoprotein PulA then makes its way to the outer membrane, which it crosses and remains anchored to via its amino-terminal fatty acid moieties. In late exponential and stationary phase of growth of laboratory cultures, PulA is released from the outer membrane and shed into the extracellular medium.

The precise mechanisms underlying the sorting of PulA to the outer membrane and how it crosses the outer membrane are not understood. However, PulA secretion from the periplasm requires the presence of 14 accessory proteins encoded by the *pulC-O* operon and *pulS* (herein referred to as the PulA-specific secretory proteins). Of all these proteins the functions of PulD and PulS are best understood. PulD is an integral outer membrane protein believed to form a channel through which PulA is secreted. PulS, also an outer membrane associated
Figure 4: The two-step secretion pathway of PulA. The first step involves the Sec proteins (SecA, B, D, E, F, and Y) and Leader peptidase II (LspA). The second step involves 14 PulA-specific proteins (PulC-O and PulS). Heavy black bar on PulA: signal peptide. Stippled circles with three vertical black bars: fatty acid modified and folded PulA. Stippled circles: folded PulA. Vertical black bars on PulA: attached fatty acids. Modified from Pugsley (1993).
lipoprotein, is required for stabilization and membrane insertion of PulD (a PulD-specific periplasmic chaperone; Hardie et al. 1996).

The most simplistic view of PulA transfer from the inner to the outer membrane would have PulA transiting the periplasm by random diffusion. Considering that the lipid modification of the amino-terminal cysteine would confer significant hydrophobicity to PulA, it would seem difficult for the protein to exist freely in the periplasmic space, which is bounded by the hydrophobic inner and outer membranes. Alternatively, the 14 essential PulA-specific secretory proteins may form a complex which serves as a periplasmic shuttle, or as a trans-periplasmic channel, through which PulA is sorted towards PulD in the outer membrane for secretion (Possot et al. 1997).

Evidence for a trans-periplasmic channel is based on and limited to the amino acid sequence similarity in alignments between PulG, H, I, and J and proteins of the type IV pilus (65-75% identical to the Pseudomonas aeruginosa pilus proteins XcpT, U, V, and W, respectively; Pugsley and Possot 1993; Hobbs and Mattick 1993). Furthermore, the PulO protein has been shown to have prepilin signal peptidase activity, is 60% identical to the P. aeruginosa XcpA peptidase, and is involved in processing of PulG, H, I, and J (Hobbs and Mattick 1993). Additionally, PulE and F are homologous to proteins required for type IV piliation (PilB and PilC, respectively; Pugsley et al. 1997). PulF is 70% identical to PilC of various Erwinia species (proposed to serve as a platform upon which fimbrial strands or pseudopilus structures form; Hobbs and Mattick 1993). Therefore, it was speculated that PulG, H, I, and J (processed by PulO) form a trans-periplasmic pseudopilin-like structure docked on PulF, through which PulA crosses the periplasm towards PulD in the outer membrane (Pugsley et al. 1997). This
hypothesis suggests that PulA would not be free in the periplasm but instead would be complexed in a large multi-protein structure.

Interestingly, PulE, which is located in the inner membrane, contains a putative nucleotide binding domain and has been shown to contain a tetracysteine motif (388CXXC...419CXXC) implicated in kinase activity (Possot and Pugsley 1994; Possot and Pugsley 1997). However, as is the case with most of the other PulA-specific secretory proteins, the exact role played by PulE is not known. Based on PulE homology to PilB (required for type IV pilus assembly in P. aeruginosa) as well as to components of membrane-associated ABC transporters required for transport of amino acids or sugars, it has been suggested that PulE may be an ATPase/kinase involved in the assembly of the other PulA-specific secretory proteins (into a pseudopilin structure; Possot and Pugsley 1994).

1.5.2 Secretion of PulA fusion proteins from K. oxytoca.

Initial experiments designed to assess the secretory signal sequences within PulA demonstrated that the amino-terminal 834 amino acids of the mature 1071 amino acid PulA protein were essential for secretion. When fused to the segment encoding the mature portion of the normally periplasmic β-lactamase gene (blaM), these sequences were sufficient to effect secretion in E. coli when the PulA-specific secretory proteins were simultaneously expressed (Kornacker and Pugsley 1990). Surface localization of the PulA:Bla chimera was monitored by immunofluorescence using anti-PulA and anti-Bla antibodies, as well as by measuring β-lactamase activity in various compartments. In these experiments 80% of the total cell-associated β-lactamase activity was exposed to the extracellular medium. More recent investigations using the PulA:Bla fusion protein have narrowed the location of the secretion
determinants in PulA to the absolute requirement for two non-adjacent regions (designated regions A and B; Sauvonnet and Pugsley 1996). Region A comprises the first 78 amino acids while region B contains amino acids 735 to 814 (in the mature PulA protein). Removal of either region A or B resulted in a 75% decrease in PulA:Bla secretion whereas deletion of both regions A and B resulted in a complete elimination of secretion. Interestingly, a PulA:Bla fusion protein containing only the PulA signal peptide, regions A and B, and the mature β-lactamase segments (designated SP-AB-BlaM) resulted in slightly reduced surface exposure of β-lactamase activity (45% versus 60%). Removal of either region A or B from SP-AB-BlaM resulted in complete elimination of secretion, whereas removal of region A or B from wild type PulA resulted in a 25% decrease in surface exposure (Sauvonnet and Pugsley 1996).

Although regions A and B appear essential for secretion of PulA hybrid proteins, there may be other determinants involved in secretion of the wild type protein. Furthermore, the nature of the heterologous protein attached to PulA may play a role in determining the effectiveness of secretion. Fusion proteins between either the entire or the first 834 amino acids of PulA and MalE (E. coli periplasmic maltose binding protein), CelZ (Erwinia chrysanthemi endoglucanase), and SacB (secreted Bacillus subtilis levansucrase) together with the above mentioned, BlaM (periplasmic β-lactamase) were tested for secretion from E. coli (Sauvonnet et al. 1995). Only the fusions between the entire PulA protein or the first 834 amino acids of PulA and either BlaM or CelZ resulted in surface exposed protein (none of the other fusion proteins were secreted). Why was a fusion protein between PulA and one periplasmic protein (PulA:BlaM) secreted whereas another (PulA:MalE) was not? Even the normally secreted SacB was not secreted as the chimeric PulA:SacB. Perhaps the overall three-dimensional structure of
the chimeric protein is an important factor in determining whether the secretory determinants of PulA are effectively presented to the PulA-specific apparatus. These recent studies indicate that we are still a long way from a complete understanding of protein secretion, especially as it relates to novel fusion proteins.

1.6 Summary.

The focus of my thesis research was to evaluate the potential of using PulA to effect the secretion of the *C. fimi* cellulases CenA, Cex, and particularly CenB as fusion proteins. With a rudimentary understanding of PulA secretion [the Kornacker and Pugsley (1990) experiments on PulA secretion were the only information on this subject that had been published], I began a series of experiments to test the use of the amino-terminal portion of PulA as a secretion determinant to effect secretion of chimeric cellulases from *K. oxytoca*. Fusion genes that would result in chimeric proteins containing the first 834 amino acids of PulA and either complete or partial sequences of *C. fimi* cellulases were constructed. These fusion genes (*pulA:cenA, pulA:cenB*, and *pulA:cex*) were introduced into *K. oxytoca* host cells encoding all the proteins required for PulA secretion. The ability of these recombinant strains to produce enzymatically active and/or secreted cellulase fusion proteins was evaluated by Western blot, enzyme assay, cell fractionation, immunofluorescence, and growth experiments.

The research data presented in this thesis represent the first step towards the larger goal of developing a biological process that could be used to convert cellulose waste material into a fertilizer. Although it was not the goal of my thesis research, it was hoped that following my results of conferring cellulase activity to a nitrogen fixing bacterium, this recombinant strategy would be developed further by others. The
introduced DNA (pulA:cenA, etc.) would be stabilized by insertion into the host bacterium's chromosome, and its regulation modified such that expression was either constitutive or internally regulated. In the long term, pilot laboratory experiments could be done prior to environmental release and assessment of the industrial applicability of a novel bacterium in a pulp and paper bio-treatment lagoon.
2.0 MATERIALS AND METHODS

2.1 Bacterial strains, media, and growth conditions.

The bacterial strains used are listed in Table I. Strains were grown at 30° C in M63 (K. oxytoca and E. coli strain pop3125; Miller 1972), LB (E. coli and K. oxytoca; Sambrook et al. 1989), or TYP (E. coli and K. oxytoca; per litre: 16 g each tryptone and yeast extract, 5 g NaCl, 2.5 g K$_2$HPO$_4$) medium. Plates were made by solidifying broth medium with 1.5% agar [or agarose (Ultrapure, BRL) for selection of growth on cellulose]. Media were supplemented with antibiotics (ampicillin: 200 μg/ml; chloramphenicol: 20 μg/ml) to ensure the maintenance of plasmids, and 0.0005% thiamine (E. coli pop3125). M63 medium was supplemented with either 0.4% (w/v) glucose, cellobiose, maltose, or glycerol as carbon sources, 0.4% (w/v) CMC (low viscosity carboxymethyl cellulose) or avicel used as the carbon source for growth on cellulose, and with 40 μg/ml histidine or 0.4% (w/v) casamino acids. Induction of secretion genes was initiated by the addition of 0.4% (w/v) maltose to either TYP or M63 medium. All cultures were incubated aerobically in glass test tubes or erlenmeyer flasks. The CenB protein (0.1 mg/ml) added to cultures of K. oxytoca was purified by FPLC/anion-exchange chromatography (Meinke et al. 1992), followed by exchange of azide-containing storage buffer for 50 mM KPO$_4$ (pH 6.9) using a P-6™ desalting column (Bio-Rad). P-6™ fractions of 0.5 ml were collected and aliquots tested for enzymatic activity by spotting onto CMC-containing plates for Congo Red plate analysis (see Section 2.22). Fractions containing CMCase activity were pooled, filtered through a 0.2 μm pore size Supor™ Acrodisc™ 25 filter (Gelman Sciences), and added to cultures.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>K. oxytoca</em> UNF5023</td>
<td><em>hisD2 hsdR1 rpsL4</em></td>
<td>Dixon <em>et al.</em> 1977</td>
</tr>
<tr>
<td><em>E. coli</em> JM101</td>
<td><em>supE thi Δ(lac-proAB)</em> [F* traD36 proAB lacIqZΔM15]*</td>
<td>Yanisch-Perron <em>et al.</em> 1985</td>
</tr>
<tr>
<td><em>E. coli</em> JM83</td>
<td><em>ara Δ(lac-proAB) rpsL (= strA) φ80 lacZΔM15</em></td>
<td>Yanisch-Perron <em>et al.</em> 1985</td>
</tr>
<tr>
<td><em>E. coli</em> HB101</td>
<td>*supE44 hsdS20 (rB−mB−) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1</td>
<td>Boyer and Rouillard-Dussoix 1969</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td><em>supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</em></td>
<td>Hanahan 1983</td>
</tr>
<tr>
<td><em>E. coli</em> C600 r−m+</td>
<td>F− e14-(mcrA−) hsdR thr-1 leuB6 thi-1 lacY1 supE44 fhuA21 mcrB</td>
<td>Bibb and Cohen <em>et al.</em> 1985</td>
</tr>
<tr>
<td><em>E. coli</em> RB404</td>
<td><em>dam-3 dcm-6 metB1 galK2 galT22 his-4 thi-1 tonA31 tsx-78 mtl-1 supE44</em></td>
<td>Brent and Ptashne 1980</td>
</tr>
<tr>
<td><em>E. coli</em> pop3125</td>
<td><em>F− araD139 Δ(lac)U169 strA relA thi (malPQ-lacZY)</em></td>
<td>Débarbouillé <em>et al.</em> 1978</td>
</tr>
</tbody>
</table>
Cultures grown for analyses of gene expression, enzyme assays, or cell fractionation were induced with 0.1 mM IPTG (for expression of the relevant genes from the plasmid-encoded lac or tac promoter) added at mid-logarithmic phase (2.0 - 2.6 X 10^8 cells/ml). Induction was routinely allowed to continue for various times (up to and including 24 hours).

2.2 Plasmids.

The plasmids used are listed in Table II, with details of novel constructions given in the Results section.

2.3 Measurement of bacterial growth.

Culture density was measured in a Klett-Summerson photometer fitted with a No. 66 (red) filter. Under these conditions 1 Klett unit is ca. 3.7 X 10^6 cells/ml.

2.4 Plasmid purification.

Routine plasmid analysis of cells was performed by the alkaline lysis method (Sambrook et al. 1989) with the following modifications. Lysozyme was omitted from the lysis solution, 3 M sodium acetate (pH 4.8) instead of the potassium acetate (pH 4.8) solution was used, and the phenol/chloroform extraction step was omitted.

Plasmid DNA used for sequencing was isolated from cells grown in LB medium using a Qiagen-tip 100 column and following the Midi™ plasmid purification protocol of the supplier (Qiagen Inc.). The DNA isolated in this way was further purified with a 30 minute dialysis.
Table II: Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>genotype and comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><code>pUC12/13</code></td>
<td>Amp^r, lacZα, Ptc, ColEI</td>
<td>Messing, 1983</td>
</tr>
<tr>
<td><code>pTZ18U/19U/18R</code></td>
<td>Phagemid version of pUC18/19</td>
<td>Mead <em>et al.</em>, 1986</td>
</tr>
<tr>
<td><code>pUC18/19</code></td>
<td>Amp^r, lacZα, Ptc, ColEI</td>
<td>Norrander <em>et al.</em>, 1983</td>
</tr>
<tr>
<td><code>pMMB207</code></td>
<td>IncQ, lacI4, Ωptac, Ωcat M13mp18 polylinker</td>
<td>Morales <em>et al.</em>, 1991</td>
</tr>
<tr>
<td><code>pCHAP231</code></td>
<td>pBR322::pulS,pulAB,pulC-O ; Source of pulA</td>
<td>Pugsley <em>et al.</em>, 1991</td>
</tr>
<tr>
<td><code>pTAL3</code></td>
<td>pTZ18R::PTIS:cenB</td>
<td>Meinke <em>et al.</em>, 1991</td>
</tr>
<tr>
<td><code>pTZEO6</code></td>
<td>pTZ18R::PTIS:ceX</td>
<td>Ong <em>et al.</em>, 1993</td>
</tr>
<tr>
<td><code>pTZ19pulA</code></td>
<td>pTZ19U::pulA</td>
<td>This study</td>
</tr>
<tr>
<td><code>pTZ19cenA</code></td>
<td>pTZ19U::cenA</td>
<td>This study</td>
</tr>
<tr>
<td><code>pTZ19pulA:cenA</code></td>
<td>pTZ19U::pulA:cenA</td>
<td>This study</td>
</tr>
<tr>
<td><code>pUC13pulA</code></td>
<td>pUC13::pulA</td>
<td>This study</td>
</tr>
<tr>
<td><code>pTZ18U:1ΔcenA</code></td>
<td>pTZ18U(polylinker Bam HI converted to Cla I::cenA)</td>
<td>Bingle <em>et al.</em>, 1993</td>
</tr>
<tr>
<td><code>pUC13pulA:cenA</code></td>
<td>pUC13::pulA:cenA</td>
<td>This study</td>
</tr>
<tr>
<td><code>pUC13pulA:cenB</code></td>
<td>pUC13::pulA:cenB</td>
<td>This study</td>
</tr>
<tr>
<td><code>pUC13pulANheI</code></td>
<td>pUC13::pulA(Nhe I at PulA codon 835)</td>
<td>This study</td>
</tr>
<tr>
<td><code>pUC13pulA:ceX</code></td>
<td>pUC13::pulA:ceX</td>
<td>This study</td>
</tr>
<tr>
<td><code>pMMBcenB</code></td>
<td>pMMB207::cenB</td>
<td>This study</td>
</tr>
<tr>
<td><code>pMMBpulA:cenB</code></td>
<td>pMMB207::pulA:cenB</td>
<td>This study</td>
</tr>
<tr>
<td><code>pUC12cenA</code></td>
<td>pUC12::cenA</td>
<td>Gift from D. Driver</td>
</tr>
</tbody>
</table>
against sterile distilled H₂O by carefully spotting the DNA solution onto the surface of a 0.025 μm pore size MF-Millipore membrane floating on the water surface.

2.5  In vitro DNA techniques.

Agarose gel electrophoresis was performed using 0.5 X TBE buffer (0.045 M Tris, 0.045 M borate, 0.001 M EDTA) and 1% agarose gels prepared in 0.5 X TBE (Sambrook et al. 1989). Electrophoresis was performed at 100 V for 60 - 90 minutes (7.5 cm length diagnostic gels) or at 20 - 30 V for 16 hours (15 cm length preparative gels). DNA was purified from agarose gels using Qiaex glass beads following the manufacturer’s directions (Qiagen Inc.). DNA restriction digestion, Klenow catalysed fill-in, and ligation were carried out according to the specifications of the supplier of the respective enzyme.

2.6  PCR mutagenesis of pulA.

An Nhe I site was introduced into pulA using PCR and the sense (5’-GATGGCCGGTAACCTCGCTG) and antisense (3’-GTCTGGGCTGCCTTCAGCATCGACTAGTACGTAG) primers. These primers correspond to nucleotides 2382-2401 and 2483-2502 in pulA, respectively, the underlined nucleotides correspond to the introduced Nhe I site, and bold nucleotides correspond to introduced sequences that do not hybridize to pulA sequences (see APPENDIX 7.4). PCR amplification was carried out in a total volume of 50 μl containing 6 ng of pUC13pulA plasmid DNA, 2.4 X 10⁻⁵ μmole of each primer, 1.5 U Taq DNA polymerase (PCR grade; BRL), 1 X PCR buffer (BRL), 1 mM MgCl₂, and a 0.2 mM mixture of all four 2’-deoxynucleotide 5’-triphosphate bases. One PCR cycle at 96⁰ C (denaturation) for 1 minute,
50° C for 30 seconds (annealing), and 72° C for 59 seconds (primer extension) was followed by 27 successive cycles of denaturation at 96° C for 5 seconds, annealing at 50° C for 30 seconds, and primer extension at 72° C for 59 seconds. The final cycle repeated the previous cycle except that incubation at 72° C was extended to 7 minutes. The resulting 137 bp PCR product was sub-cloned into the *pulA* gene by digestion, and subsequent ligation, at the *Bst* EII and *Bcl* I restriction sites (see APPENDIX 7.4) and called *pulA*Nhol. The predicted translation product of the *pulA*Nhol gene would be a protein truncated immediately following the introduced *Nhe* I restriction site (see APPENDIX 7.4). The introduction of an *Nhe* I site without additional mutation in *pulA*Nhol was confirmed by DNA sequencing (Section 2.7) using the PCR primers outlined above.

2.7 DNA sequencing.

Dialysed plasmid DNA (0.8 pmol) was denatured by a 10 minute incubation in 0.2 N NaOH/0.001 M EDTA, precipitated by the addition of 1/10 volume 3 M sodium acetate (pH 4.8) and 2.5 volumes 95% ethanol and, following a 5 minute incubation, the DNA was collected by centrifugation at 15 000 X g. The DNA pellet was washed in 70% ethanol, pelleted again, and dried under vacuum. The dried DNA pellet was reconstituted with 2.8 μL DMSO, 2 μL Pharmacia annealing buffer, 2 μL sequencing primer (20 mer 5'-GATGGCCGGTAACCTCGCTG specific for *pulA*), and distilled H₂O to 14 μL, heated to 67 °C for 2 minutes, cooled slowly to ≤ 37° C, and centrifuged briefly to collect any condensate. DNA prepared in this manner was sequenced using (α-35S)dATP or (α-32P)dATP (NEN-Dupont), the Pharmacia LKB Biotechnology T7Sequencing™ kit and Deaza T7Sequencing™ mixes, and the sequencing reactions were electrophoresed on polyacrylamide gels as described in the Pharmacia T7Sequencing™ kit instructions with the
following modifications: 0.5 X TBE buffer, 42% urea, 7.6% acrylamide, and 4 mm spacers were used. Reactions prepared with (α-\(^{32}\)P)dATP were run out on gels immediately and urea was not extracted from gels run with (α-\(^{35}\)S)dATP prepared samples before autoradiography.

The DNA sequences of \(pulA, pulB, pulC-O, pulS, cenA, cex,\) and \(cenB\) were obtained using the GenBank accession numbers X52181/M32702, M29097, M32613/M24118/X14954, M29097, M15823, L11080, and M64644, respectively.

2.8 Cell transformation.

\(E.\ coli\) and \(K.\ oxytoca\) cells were transformed using the CaCl\(_2\) method (Sambrook \(et\ al\). 1989) or by electroporation using a Bio-Rad Gene Pulser\(^\text{TM}\) set at 200 \(\mu\Omega\), 25 \(\mu\)F, and 2.5 kV and 2 cm electrode cuvettes (Bio-Rad).

Cells for electroporation were grown aerobically to mid-logarithmic phase (~200 Klett units, 1.1 \(\times\) 10\(^9\) cells/ml) at 37\(^\circ\) C in an erlenmeyer flask filled to 20% of its nominal volume. The cells were pelleted by centrifugation at 10 000 X g for 10 minutes at 4\(^\circ\) C and resuspended in an equal volume of ice-cold sterile distilled H\(_2\)O. Cells were pelleted three times, resuspending in sequence, first in 0.5 volumes ice-cold sterile distilled H\(_2\)O then in 1/20 volume ice-cold sterile 10% glycerol (made in H\(_2\)O), followed by the final resuspension in ice-cold sterile 10% glycerol to a concentration of 1/1000 of the original culture. The electrocompetent cells were aliquoted into 40 \(\mu\)L volumes, flash frozen in a dry-ice/ethanol bath and stored at -80\(^\circ\) C.

Electrocompetent cells were thawed on ice prior to use. Five \(\mu\)L of DNA (diluted 1/20 in distilled H\(_2\)O) were mixed with the thawed
competent cells. Electroporation using the above settings routinely took 4 milliseconds. Electroporated cells were immediately transferred to test tubes containing 1 ml of TYP broth and incubated for 2 hours at 30° C before spreading onto selective medium.

2.9 In vitro protein methods.

Cells from TYP grown cultures were pelleted by centrifugation at 12 000 X g for 10 minutes, washed in fresh medium, and resuspended in 1/50 volume 50 mM Tris-Cl (pH 6.8). Cells prepared in this manner were analysed for total protein content (washed cell pellet resuspended in 1 X Laemmli loading buffer; Laemmli 1970), soluble intracellular protein content (as described below), or fractionated into cytoplasmic, periplasmic, inner and outer membrane fractions (as described in Section 2.15). Intracellular proteins were isolated from cells that were disrupted by two passes through a French pressure cell with pressure maintained at 200 psi during cell breakage (Power laboratory press, American Instrument Co., Inc.). Disrupted cells were immediately cooled on ice and then cleared of unbroken cells by centrifugation at 1000 X g for 10 minutes. Samples prepared as described above were used in endoglucanase (CMCase) assays (Section 2.21) and/or SDS-PAGE (Section 2.10).

Ultrafiltration using a Centricon-100 concentrator unit (Amicon) with a molecular mass cut off of 100 kDa was used as specified by the manufacturer to separate proteins of 100 kDa (flow-through) and 200 kDa (retentate). A Centricon-10 (molecular mass cut off of 10 kDa) concentrator unit was used to concentrate protein samples.

Proteins containing a cellulose binding domain were purified from heterogeneous mixtures by reversible adsorption to insoluble
cellulose (avicel) as described previously (Meinke et al. 1991), with the modification that proteins were eluted from avicel by boiling for 5 minutes in 1 X Laemmli (Laemmli 1970) protein loading buffer.

2.10 SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

The Laemmli buffer system was used for protein separation by SDS-polyacrylamide gel electrophoresis (Laemmli 1970). Except where specified, the separating gel was 7.5% acrylamide (acrylamide/bis 36.5:1) and the stacking gel was 4%. Routinely, 0.75 mm thick slab gels were prepared using either the Bio-Rad Protean™ II or Mini-Protean™ II systems. The electrophoresis conditions used were those specified by the manufacturer. This acrylamide concentration allowed effective separation of proteins between 200 and 40 kDa. Broad range pre-stained molecular mass markers (BRL or NEB) were used as described by the supplier. Proteins were visualized by staining the gel with 0.25% Coomassie Brilliant Blue (R or G; dissolved in 40% methanol/10% acetic acid) and then destaining by soaking the gel in 40% methanol/10% acetic acid. Gels were preserved by soaking for 24 - 48 hours in 3% glycerol (in H₂O) and then air dried between H₂O equilibrated sheets of cellophane.

2.11 Two-dimensional (2-D) gel electrophoresis.

Proteins isolated from induced cells as described above (Section 2.9) and purified by avicel binding were sequentially precipitated in acetone 3 times and resuspended in isoelectric focusing (IEF) sample buffer [9.5 M urea, 2% (v/v) Nonidet P40, 4.1% acrylamide (acrylamide/bisacrylamide 30.8% T, 2.6% C), 5 mM CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) prepared in
Milli-Q (Millipore) purified H₂O. Proteins were separated by IEF in the first dimension following the protocol outlined by Millipore (Millipore, Investigator™ 2-D electrophoresis system) using 1 mm diameter tube gels and a pH gradient of 3 - 10. Gel tubes were layered onto the top of 7.5% SDS-polyacrylamide slab gels (1 mm thickness) for electrophoresis in the second dimension as outlined in Section 2.10.

2.12 Western blotting.

Transfer of protein to nitrocellulose membranes from Laemmli SDS-PAGE or 2-D-gels was accomplished using the Towbin buffer system (Towbin et al. 1979) and either the Bio-Rad Trans-Blot™ or Mini Trans-Blot™ electrophoretic transfer apparatus. Transfer was accomplished at 60 V for a maximum of 1 hour with buffer cooling. Nitrocellulose membranes (Protran™; Schleicher & Schuell) were first hydrated in distilled H₂O and then equilibrated in Towbin transfer buffer for at least 15 minutes before preparing the gel/membrane transfer sandwich. The efficiency of transfer was monitored by Coomassie staining of the gel. Proteins were detected using anti-CenB polyclonal antibodies (described below) diluted 1:40 000, horseradish peroxidase-conjugated anti-rabbit secondary antibodies from donkey (Amersham) diluted 1:40 000, and the ECL™ chemiluminescence detection system (Amersham). X-ray film (Kodak X-OMAT™AR) was exposed to the Western blot from 5 seconds to 5 minutes depending on the amount of immunoreactive protein loaded.

Anti-CenB polyclonal antibodies (gift from the UBC Cellulase group) were isolated from rabbits injected with CenB in its native conformation. Rabbit serum containing anti-CenB antibodies were preabsorbed with cell extracts of K. oxytoca (pMMB207) as described below in order to remove all non-CenB specific immunoreactive species
prior to use. A 5 ml TYP culture of *K. oxytoca* (pMMB207) was grown overnight at 30° C. Cells were pelleted at 15 000 X g, resuspended in 3 ml 50 mM KPO4 (pH 7.0), and disrupted by two passes through a French pressure cell (see Section 2.9). The cell extract was separated from unbroken cells by centrifugation for 5 minutes at 1000 X g, mixed in equal volumes with antiserum, and incubated overnight with constant agitation at 4° C. Antiserum - cell extract mixtures were clarified by centrifugation at 15 000 X g for 15 minutes, the antibody-containing supernatant removed and aliquoted into Eppendorf tubes, and stored at -20° C.

Proteins detected by Western blot analysis were quantified using ImageQuant™ V1.1 (Molecular Dynamics). The relative protein concentration of the various immunoreactive species was estimated by comparing the measured pixels within defined volumes. Specific amounts of anti-CenB immunoreactive species were determined by comparing the measured pixel volumes to those of known amounts of purified CenB detected on the same western blot. In all cases a signal-free area of the gel was used for a background correction, only signals on the same gel were compared to each other, and a dilution series was analysed to remain within the linear relationship of the signal (this varied with exposure time and amount of protein loaded on the gel, such that as the signal intensified the quantitation reached a plateau region resulting in poor correlation between signal and amount of protein measured).

2.13 N-terminal sequencing.

Protein samples for N-terminal sequencing by Edman degradation (Matsudaira 1987) were purified by avicel binding and separated by electrophoresis as described (Sections 2.9 and 2.10) with the following modifications. Proteins bound to avicel were eluted by boiling
in 1 X Laemml loading buffer (Laemmli 1970) for two minutes, all electrophoresis and staining/destaining solutions were made using Milli-Q purified water, and the polyacrylamide gels were polymerized at least 24 hours prior to use to minimize the amount of acrylamide monomer (N-terminus blocker). Proteins separated by electrophoresis were transferred to either polyvinylidene difluoride membranes (PVDF; Westran™; Schleicher & Schuell) using the CAPS buffer system (3-cyclohexylamino-1-propanesulfonic acid; Matsudaira 1987) or nitrocellulose using the Towbin system (as described in Section 2.12), however the polyacrylamide gel was not equilibrated in transfer buffer. PVDF membranes were wetted in 100% methanol (at least 5 seconds) and then equilibrated in transfer buffer (at least 5 minutes) before use. Following transfer of proteins to membranes, the membranes were rinsed twice in Milli-Q purified water, stained 1 - 2 minutes in 0.025% (w/v) Coomassie blue, destained in 50% methanol/Milli-Q water until protein bands were visible (5 - 15 minutes), rinsed several times with Milli-Q purified water (5 - 10 minutes each), and then air dried. Protein bands to be sequenced were excised from the membrane with a scalpel, the ends of the proteins deblocked with trifluoroacetic acid, and the protein sequenced using an Applied Biosystem automatic sequencer operated by the UBC Peptide/Protein Sequencing Nucleic Acid-Protein Service Unit.

2.14 Zymogram analysis.

Protein samples for zymogram analysis were prepared in Laemmli protein loading buffer which lacked 2-mercaptoethanol and were incubated at room temperature for at least 5 minutes prior to loading into wells of the gel. Once SDS-PAGE was completed (gels were run for 24 hours at 8 mA/gel), the gel was soaked in 50 mM KPO₄ (pH 7.0) buffer with 4 buffer changes for 30 minutes each. The gel was then layered over a 1% agarose/0.4% low viscosity CMC gel [in 50 mM KPO₄...
This gel sandwich was enclosed in a moisture retaining container and incubated overnight at 30° C. The agarose gel underlay was stained overnight in 1 mg/ml Congo red and destained in 1 M NaCl/50 mM KPO₄ (pH 6.9) until clearings were detected. To enhance the contrast between zones of clearing and the Congo red-stained gel background, some gels were further treated by soaking in 10% acetic acid. Zymograms were dried between layers of cellophane as described above (Section 2.10). The areas of clearing were quantified as described for Western blot quantification. As with quantification of Western blots, quantification of zymograms is also subject to saturation kinetics, and so this was taken into account as described in Section 2.12.

2.15 Cell fractionation.

Cells were fractionated into periplasmic, cytoplasmic, inner membrane, and outer membrane enriched fractions in the following manner. Cells from 100 ml cultures were separated from the growth medium by centrifugation at 12 000 X g for 10 minutes at 4° C. The cell-free culture supernatant fluids were kept as the cell-free “culture medium” fraction. The cell pellet was resuspended in 8 ml 20% (w/v) sucrose, 30 mM Tris-Cl (pH 8.0). One tenth of this cell suspension was removed and saved as the “unfractionated” control sample. The “periplasmic” enriched fraction was prepared by modification of the osmotic shock protocol (Neu and Heppel 1965), such that volumes were proportionally reduced to 1/10th of the published volumes. Briefly, cell pellets were resuspended in 8 ml of 20% sucrose (w/v), 0.03 M Tris-Cl (pH 8.0) and 2 ml 5 mM EDTA. After mixing on a rotating shaker at 180 RPM for 10 minutes at room temperature the cells were pelleted by centrifugation at 12 000 X g for 10 minutes at 4° C. Cells were resuspended in 10 ml ice-cold distilled H₂O, mixed as described above in
an ice bath for 10 minutes. Cells were again pelleted, resuspended in ice-cold distilled H₂O as described above, and pelleted to remove sphaeroplasts. Both H₂O supernatants were pooled, concentrated using a Centricon-10 filter (molecular mass cut off of 10 kDa), and assayed for the presence of periplasmic proteins (see Section 2.18).

The sphaeroplasts resulting from the osmotic shock procedure described above were resuspended in 20% sucrose (w/v)/10 mM Tris-HCl (pH 8.0) and frozen overnight at -20° C. The cells were thawed on ice, DNaseI added to 50 μg/ml, and incubated for 15 minutes at room temperature before being broken by two passes through a French pressure cell as described above (Section 2.9). The lysed cells were immediately placed on ice, centrifuged at 1000 X g to remove unbroken cells, and the supernatant liquid centrifuged at 90 000 X g in an ultracentrifuge for 60 minutes. The resulting supernatant contained cytoplasmic proteins and was collected as the “cytoplasmic” fraction. The resulting pellet, containing the inner and outer membrane fractions, was resuspended in 20% sucrose (w/v)/10 mM Tris-HCl (pH 8.0) and layered onto the top of a 4 step sucrose gradient [70%, 64%, 58%, and 52% sucrose (w/v)/10 mM Tris-HCl (pH 8.0)] and centrifuged in a SW41 rotor for 16-18 hours at 39 000 RPM and 4° C. Fractions were collected by dripping the gradient from a hole made at the bottom of the tube. The lowest white-coloured band was collected as the “outer membrane” fraction, and the uppermost yellowish band was collected as the “inner membrane” fraction.

Each fraction was assayed for β-galactosidase, alkaline phosphatase, NADH dehydrogenase, and 2-keto-3-deoxyoctonic (KDO) acid as markers for the cytoplasm, periplasm, inner and outer membrane fractions, respectively (as described below).
2.16 Outer membrane protein extraction.

Outer membrane fractions were treated with octyl-polyoxyethylene (octyl-POE) to aid in distinguishing between genuine membrane proteins and inclusion bodies that could have co-purified with membrane particles in this procedure (Pugsley and Possot 1993; Piers et al. 1993). This method is based on the observation that proteins are readily extracted from the outer membrane by solubilization with octyl-POE whereas inclusion bodies are less soluble in this detergent (Garavito and Rosenbusch 1986). In brief, a 1 ml membrane sample was brought to 0.5% (v/v) octyl-POE and incubated at 37° C for 1 hour. Membrane particles and inclusion bodies were separated from solubilized proteins by centrifugation at 120 000 X g for 75 minutes. Membrane pellets were resuspended in 1 ml 10 mM Tris-Cl (pH 8.0), 50 mM EDTA, 3% (v/v) octyl-POE. Following incubation at 37° C for 1 hour the insoluble fraction was separated from the solubilized protein by centrifugation at 120 000 X g for 75 minutes. The membrane pellet was sequentially treated 4 more times in this manner with 3% (v/v) octyl-POE. The final insoluble pellet was considered to contain proteins in inclusion bodies and membrane lipids. Each fraction collected was examined separately by Western blot analysis following SDS-PAGE.

2.17 β-galactosidase assays.

β-galactosidase activity was measured in unfractionated (intact cell) or cell fractions (ca. 1 - 5 X 10^9 cells or and equivalent volume of the original sample) as described (Miller 1992). In brief, samples to be assayed were diluted in 1 ml Z buffer (Miller 1992), 0.2 ml of 4 mg/ml ONPG (prepared in Z buffer) was added, and incubated at 37° C. When a perceptible yellow colour developed due to the release of o-nitrophenol the reaction was stopped by the addition of 0.5 ml 1.0 M
Na$_2$CO$_3$, elapsed time recorded, reaction mixtures cleared of particulate material by centrifugation at 15 000 X g for 7 minutes, OD measured at 410 nm and 550 nm, and activity calculated and expressed as previously described (Miller units; Miller 1992). If no perceptible yellow colour developed within 40 minutes of incubation the reaction was stopped by the addition of Na$_2$CO$_3$. Protein concentrations were determined using a Lowry procedure modified for membrane proteins (Peterson 1983).

2.18 Alkaline phosphatase (PhoA) assays.

Alkaline phosphatase (samples containing ca. 1 - 5 X 10$^9$ cells or an equivalent volume of the original sample) was assayed as described (Manoil 1991). In brief, samples to be assayed were diluted in 1 ml 1.0 M Tris, 0.1 mM ZnCl$_2$ (pH 8.0), 0.1 ml of 0.4% Sigma 104™ Phosphatase Substrate (prepared in Tris/ZnCl$_2$) was added, and incubated at 37° C. When a perceptible yellow colour developed due to the release of p-nitrophenol the reaction was stopped by the addition of 120 |L 1.0 M KH$_2$PO$_4$/0.5 M EDTA. If no perceptible yellow colour developed within 40 minutes of incubation the reaction was stopped by the addition of KH$_2$PO$_4$/0.5 M EDTA. Reaction mixtures were centrifuged at 15 000 X g for 7 minutes to pellet particulate material before measuring the OD at 410 nm and 550 nm, and activity calculated and expressed as previously described (Miller units; Miller 1992). Protein concentrations were determined using a Lowry procedure modified for membrane proteins (Peterson 1983).

2.19 NADH dehydrogenase assays.

Intact cells (ca. 1 - 5 X 10$^9$) cells or cell fractions (volume of sample equivalent to that originally occupied by 1 - 5 X 10$^9$ intact cells) were assayed by monitoring the rate of NADH oxidation by continuous
spectrophotometry at 340 nm as previously described (Osborn et al. 1972). Samples to be assayed were added to cuvettes containing 50 mM Tris-Cl (pH 7.5) and 0.2 mM dithiotreitol. The background rate of endogenous NADH oxidation was monitored for 30 seconds before the addition of NADH (to a final concentration of 0.12 mM). NADH dehydrogenase activity was determined from the initial net rate of exogenous NADH oxidation and expressed as AOD/minute/mg protein. Protein concentrations were determined as described above.

2.20 2-Keto-3-deoxyoctonic acid (KDO) assays.

KDO (an outer membrane marker) was assayed according to a method modified from Weissbach and Hurwitz (Weissbach and Hurwitz 1959). The lipopolysaccharides in a 50 μL sample of each fraction (ca. 1 - 5 X 10⁹ cells starting material) were hydrolysed to their component sugars by the addition of 50 μL 0.5 M H₂SO₄, mixing, and heating for 15 minutes at 100°C. Once the samples had cooled to room temperature, 50 μL of 0.1 M H₅IO₆ was added, the mixture vortexed, incubated 10 minutes at room temperature to allow the oxidation of KDO to formylpyruvic acid, and 200 μL 0.42 M NaAsO₂ (in 0.5 M HCl) was added to stop the reaction. To the reaction mixture was added 800 μL 4.2 mM thiobarbituric acid, the sample vortexed, heated for 10 minutes at 100°C, and cooled to room temperature. The resulting red chromogen (the product of a reaction between formylpyruvic acid and thiobarbituric acid) was extracted by adding 1.5 mL of an acid/butanol solution (95 mL n-butanol acidified with 5 ml concentrated HCl), vortexing, centrifugation for 5 minutes at 1000 X g. The upper butanol layer was removed and its optical density measured at 508 and 552 nm. A KDO standard curve prepared at the same time indicated that an OD₅₅₂ minus OD₅₀₈ of 21 corresponded to 1 μmole of KDO (very close to the
previously published value of 19/1 μmole KDO; Osborn 1963). Protein concentrations were determined as described above and KDO content expressed as mg KDO/mg protein.

2.21 Endoglucanase (CMCase) assays.

Cultures grown for CMCase assays were concentrated 25-fold in 50 mM KPO₄ (pH 7.0) buffer and divided into two samples. One portion was lysed in a French pressure cell (as described in Section 2.9) and the other was left intact for the determination of cell-surface exposed enzymatic activity. Potential enzymes from samples of cell-free culture medium were separated from medium components (eg. low molecular mass carbohydrates) that interfered with the DNS assay by size-exclusion chromatography using an Econo-Pac™ 10DG Column (Bio-Rad) according to the manufacturer’s desalting protocol.

CMCase activity was assayed by measuring the amount of reducing sugars produced from the hydrolysis of low viscosity CMC using the dinitrosalicylic acid (DNS) method (Miller 1959) with one modification. BSA was omitted from the enzyme dilution buffer as well as the CMC substrate solution. Reproducibility of this assay was maintained by careful preheating of the CMC substrate solution to 37° C and using an Eppendorf™ Repeater™ Pipettor to deliver the substrate to the reaction tubes.

In brief, 250 μL of an enzyme-containing solution was incubated with 500 μL of substrate CMC [4% CMC, 50 mM KPO₄ (pH 6.9)] at 37° C for 30 minutes. DNS reagent [1% DNS, 1% NaOH, 0.2% phenol, 0.05% Na₂SO₃, 20% C₄H₄KNaO₆, and 0.01% NaN₃ (as preservative)] was added to stop hydrolysis and oxidize the aldehyde group of the reducing sugars. The resulting carboxylic forms of the
sugars decomposed upon heating at 100° C, to coloured compounds and the concentration of these compounds was quantified by measuring optical density at 550 nm using specific blanks (equivalent volume of enzyme solution added after adding DNS reagent) and correlated to glucose content by comparison to a glucose standard curve. Protein content was determined as described above. CMCase activity (µmoles glucose-equivalents released/minute/ml) = [(OD_{550})(X)]1000/[(µL enzyme sample)(30 minutes)], where X = µmoles glucose corresponding to an OD_{550} of 1.0 as measured from a glucose standard curve.

Lysis and outer membrane disruption of intact cells in the DNS assay was monitored by removing aliquots from the reaction mixtures after the 30 minute incubation period, pelleting the cells by centrifugation at 15 000 x g for 10 minutes, and assaying for β-galactosidase (cell lysis) and PhoA (outer membrane disruption) activity as described (Section 2.17 and 2.18, respectively). β-galactosidase and PhoA activity measured from CMCase reaction mixes prepared using intact cell samples was compared to activity in corresponding samples prepared from French pressed cell lysates.

2.22 Congo red plate assay.

Colonies expressing cellulose endoglucanases were identified using the Congo red plate assay as described (Teather and Wood 1982). Agar plates were prepared with media (described in Section 2.1) and supplemented with 1% CMC (strains expressing CenA and derivatives) or 0.4% CMC (for strains expressing CenB and its derivatives) and 0.1 mM IPTG (for induction of plasmid-encoded genes). Routinely, 5 to 10 µL of an overnight broth culture was spotted onto the agar surface and the liquid allowed to soak into the medium. Following a 24 - 48 hour incubation period at 30° C, the resulting bacterial growth was washed off
with distilled water, the agar surface stained with a Congo red solution (0.5 mg/ml) for 30 - 60 minutes, and then destained as described for zymograms (see Section 2.14). Endoglucanase activity was detected as a zone of clearing (halo) around the area formerly occupied by bacterial cells (Congo red has a high binding affinity for intact, but not hydrolysed, CMC molecules). To increase the contrast between zones of clearing and the background, destained plates were washed with 10% acetic acid for ca. 5 minutes.

2.23 Methyl-umbelliferyl cellobioside (MUC) plate assay.

Agar plates were prepared as described in Section 2.22 and supplemented with 100 µM MUC (Sigma). The plates were spotted with bacterial suspensions and incubated as described above in Section 2.22. Exoglucanase activity was detected as fluorescence when the plate was exposed to UV light.

2.24 N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) mutagenesis.

*K. oxytoca* (pMMBpulA:cenB) cells were mutagenized with MNNG (13.2, 50, 100 and 1000 µg/ml) as described (Miller 1992). Briefly, a fresh overnight grown TYP culture of *K. oxytoca* (pMMBpulA:cenB) was subcultured by 1/50 dilution in 30 ml TYP medium, incubated at 30°C for 2 hours without agitation, and then incubated with aeration until a density of 2 X 10^8 cells/ml in a shaking water bath. Cells were washed twice in 0.1 M citrate buffer (Miller 1992), resuspended in 0.5 volumes citrate buffer, and divided into 6 aliquots of 2.5 ml. MNNG was added and aliquots incubated for either 45, 30, 15, 10, and 5 minutes with gentle shaking (one aliquot not treated with MNNG (0 minutes) was used as the non-mutated control). Following mutagenesis, cells were washed twice in 2 volumes 0.1 M
phosphate buffer (Miller 1992) and resuspended in 2.5 ml phosphate buffer. Each time point sample was titred by plating on TYP $10^{-4}$ and $10^{-5}$ dilutions (mutagenized samples) and $10^{-5}$ and $10^{-6}$ dilutions (control samples) to determine cell survival during mutagenesis. Overnight cultures were generated by diluting each time point into 50 ml TYP, incubating overnight at $30^\circ$ C, and dilutions ($10^0$ - $10^{-2}$) plated to determine mutation frequency to rifampicin resistance. Cells from each time point were also used to inoculate liquid and solid medium (see Section 2.1) for the selection of growth on cellulose.

2.25 Detection of cell surface proteins by immunofluorescence.

Cell surface proteins were detected by immunofluorescence using intact cells either immobilized and fixed on poly-L-lysine coated slides or in solution. Two cultures each of *K. oxytoca* (pMMB207), *K. oxytoca* (pMMBcenB), and *K. oxytoca* (pMMBpulA:cenB) were grown in 50 ml TYP. One culture of each pair was supplemented with 0.4% maltose for induction of PulA-specific secretion proteins. Following 5 hours of induction with 0.1 mM IPTG, $1 \times 10^9$ cells from each sample were washed twice and resuspended in PBS (to a final concentration of $1 \times 10^9$ cells/ml).

Cells were immobilized by depositing 30 μL of each sample onto three poly-L-lysine coated slides (Sigma Poly-Prep™ Slides), and allowing to air dry completely. Cells dried on slides were fixed by incubation in 0.7 ml 3% paraformaldehyde (pH 7.4) for 20 minutes at room temperature [3 g of paraformaldehyde powder were added to 100 ml PBS (phosphate buffered saline; Sambrook *et al.* 1989). While stirring and heating on a hot plate in a fume hood, 4 drops of 1.0 M NaOH were added to aid dissolution, followed by 10 μL 1.0 M CaCl$_2$ and
10 μL 1.0 M MgCl₂. The solution was cooled to room temperature, suction filtered through a 0.45 μm Millipore filter, and frozen at -20° C in 20 ml aliquots. Thawed paraformaldehyde was not refrozen. Labelling of cells with primary and secondary antibodies and sealing of slides in SlowFade™ reagent (Molecular Probes) were as described (Sun et al. 1998) with several modifications. Only one slide of each triplet was treated with lysozyme while the others were incubated with PBS. Anti-CenB and goat anti-rabbit-FITC (Jackson ImmunoResearch Laboratories Inc.) labelled antibodies were used at a titre of 1/100 and 1/20 respectively. Cells treated with lysozyme were included as a positive control for labelling and detection, while anti-CenB antibodies omitted from a second slide (of each triplet) resulted in a control for non-specific labelling by the goat anti-rabbit FITC conjugate.

Cells labelled in solution were grown and prepared as described above for immobilized and fixed cells. Once cells were resuspended, 30 μL were removed into an eppendorf tube to which was added 10 μL of a 1/100 dilution of anti-CenB antibodies (diluted in PBS/2% BSA). Following incubation for 1 hour at 4° C, cells were washed in 1 ml PBS, resuspended in 30 μL PBS, and 10 μL of a 1/20 dilution of goat anti-rabbit FITC conjugate were added (diluted in PBS/2% BSA). Following incubation for 45 minutes at 4° C (in the dark), cells were washed three times in 60 μL PBS, and resuspended in 30 μL SlowFade™ reagent (Molecular Probes).

Cells labelled as described above (+/- fixing in paraformaldehyde) were sandwiched between microscope slides and cover slips and fluorescence detected with a Zeiss fluorescence microscope using an exciter filter of 485 nm and a barrier filter of 510 nm. Both phase contrast and UV-fluorescent images were photographed with an attached Olympus OM2 camera and TMAX400 black-and-white
film [Kodak; phase images were exposed to film using the automatic exposure setting of the camera (ca. 1/3rd second) and fluorescent images were exposed to film for 12 - 50 seconds (fixed cells) or 12 - 100 seconds (unfixed cells)].

2.26 Pullulanase assay.

Pullulanase activity was assayed using the red-pullulan chromogenic substrate for α-1,6-amylases (Procion red complexed with the sugars in pullulan; Megazyme). Cell extracts and intact cells were prepared as described for CMCase assays (Section 2.21). Enzyme-containing samples were diluted in reaction buffer [0.2 M sodium acetate, 1 mg/ml BSA; (pH 5.0)] to a final volume of 1 ml, 0.5 ml 2% red-pullulan (prepared in 0.5 M KCl) was added, and incubated for 20 minutes at 40°C. The reaction was stopped and high molecular weight red-pullulan precipitated by the addition of 2.5 ml 95% ethanol, the solution stirred and then left to settle for 10 minutes at room temperature, and large particles removed by centrifugation at 1000 X g for 10 minutes. The absorbance of light at 510 nm of the supernatant fluids containing solubilized chromophore (Procion red) was measured against blanks for each assay reaction (prepared by adding ethanol to enzyme extracts before adding the substrate red-pullulan). Pullulanase activity was expressed as absorbance units (AU)/mg total protein (as determined using the modified Lowry method of Peterson; Peterson 1983).
3.0 RESULTS

3.1 Construction of fusion genes.

Four independent fusions were made between the *K. oxytoca* pulA gene and the *C. fimi* cellulase-encoding genes cenA, cex, and cenB.

3.1.1 Construction of pulA:cenA fusion genes.

The first of two pulA:cenA fusion genes (designated pTZ19pulA:cenA; Figure 5) was constructed by fusion of the pulA EcoRV site to the cenA PvuII site (see APPENDIX 7.1 and 7.4). The resultant 3235 bp translationally in-frame fusion gene could be transcribed from either the subcloned pulA promoter controlled by the maltose regulon (maltose is the nominal inducer; Michaelis *et al.* 1985) or by the plasmid-encoded lac promoter controlled by LacI and the gratuitous inducer IPTG. The resultant protein would be 1078 amino acids long, would contain the 19 amino acid wild type PulA signal peptide, and is predicted to have a molecular mass of 124 kDa.

The second pulA:cenA fusion gene was created by fusion between the pulA Bcl I and the cenA BamHI sites [designated pUC13pulA:cenA; Figure 6; (see APPENDIX 7.1 and 7.4)], and contains an additional 422 bp of pulA and 8 bp of cenA sequences as well as the sequences described for the fusion in pTZ19pulA:cenA. The resultant 3667 bp pulA:cenA chimera could be transcribed from either the pulA or lac promoter as described above. The resultant PulA:CenA protein would be 1221 amino acids long with a predicted molecular mass of 140 kDa and would contain the wild type PulA signal peptide.
Figure 5: Outline of *pulA:cenA* fusion gene construction at the *pulA* EcoR V site. Expression of the fusion gene is controlled by the subcloned *pulA* promoter (divergent arrowheads and shorter single arrowhead) or by the pTZ19 encoded *lac* promoter (taller single arrowhead). Underlined bases represent codons at the site of gene fusion. The *Pvu* II site in *cenA* starts at base pair 235 (48th codon of the 112 codon CBD of the mature protein; see APPENDIX 7.1) of the 1350 bp gene. The *EcoR* V site starts at base pair 2119 in the 3273 bp *pulA* gene (see APPENDIX 7.4). Plasmid pTZ19cenA was created by subcloning a 1.6 kb *Sst* I fragment containing *cenA* from pUC12cenA and orientation checked by double digestion with *Xba I* and *Pvu* II. The open rectangles with adjoining arrowheads indicate genes and the direction of transcription, respectively.
pTZ19U

pCHAP231
23 kb

pTZ19pulA'
5.4 kb

EcoR I
Kpn I

Kpn I
purify 2.8 kb fragment

Kpn I
purify 2.5 kb fragment

pTZ19pulA':cenA
6.6 kb

5' CCG GAT CTG TGG 3'

pTZ19cenA
4.5 kb

EcoR I
Xba I

EcoR V
Pvu II

Xba I & EcoR V
purify 5.3 kb fragment

Xba I & Pvu II
purify 1.3 kb fragment

Kpn I
EcoR V

pulC-O

Kpn I

pulA-B
Figure 6: Outline of \textit{pulA:cenA} fusion gene construction at the \textit{pulA} \textit{Bcl} I site. Expression of the fusion gene is controlled by the pUC13 encoded \textit{lac} promoter (single bent arrowhead) or the subcloned \textit{pulA} promoter (divergent arrowheads). Underlined bases represent codons at the site of gene fusion. The resulting plasmid pUC13pulA:cenA also contains the first 176 bp of the 857 bp gene \textit{pulC}. The \textit{Bcl} I site starts at base pair 2543 in the 3273 bp gene \textit{pulA} (see APPENDIX 7.4). The \textit{BamH} I site in \textit{cenA} starts at base pair 227 (45th codon of the 112 codon CBD of the mature protein) of the 1350 bp gene (see Appendix 7.1). The open rectangles with adjoining arrowheads indicate genes and the direction of transcription, respectively.
The diagram illustrates the generation of a plasmid vector from pUC13 and pulA-pre, resulting in pUC13pulA:cenA.

1. **pUC13** is digested with **BamH I** and **Hinc II** enzymes, and the **Sst I** enzyme is used to purify a 6.6 kb fragment, which is ligated into the **Sst I** site of **pCHAP231**.

2. **pCHAP231** is a circular plasmid with 23 kb in length containing the **pulA-B** and **pulC-O** regions.

3. **pUC13pulA-pre** is digested with **Sst I** to create **pUC13pulA** with an 8.9 kb fragment containing the **pulA** gene.

4. **pUC13pulA** is further digested with **Bgl II** and **Bsa A I** enzymes, creating 6.4 kb and 1.3 kb fragments, respectively.

5. **pUC13pulA:cenA** is generated by ligating the 6.4 kb fragment into the **Hind III** site of **pTZ18U** containing 1ΔcenA, resulting in a 7.7 kb plasmid.

The 5' and 3' sequences for the **pulA** gene are also shown: 5' GAC ATG ATC CAG CAG 3'.
The plasmid pUC13pulA:cenA also contains the 5' 176 bp of the 857 bp pulC gene upstream of pulA, and is transcribed in the opposite direction from another maltose regulated promoter (Chapon and Raibaud 1985).

Both pTZ19pulA:cenA and pUC13pulA:cenA encode the wild type pulA ribosome binding site and ATG translation start codon. In both cases the predicted protein would contain ca. 40% of the 112 amino acid CenA CBD (64 amino acids in pTZ19pulA:cenA and 67 amino acids in pUC13pulA:cenA), and that portion of PulA originally thought to be sufficient for secretion of fusion proteins (706 amino acids of PulA in pTZ19pulA:cenA and 847 amino acids of PulA in pUC13pulA:cenA; Kornacker and Pugsley 1990; see Figure 9A). The junctions of both fusion genes were sequenced and found to be translationally in-frame (data not shown).

3.1.2 Construction of the fusion gene pulA:ces.

The Nhe I sites used for creating the pulA:ces fusion gene (Figure 7 and APPENDIX 7.2 and 7.4) were introduced into pulA and ces by PCR mutagenesis as described in Section 2.6. The resulting 3835 bp pulA:ces chimera may be transcribed from either the pulA or lac promoter as described above, and encodes the wild type pulA ribosome binding site and translation start codon. The predicted PulA:Cex protein would retain the 443 amino acids of the mature Cex protein and 834 amino acids of PulA, and has a predicted molecular mass of 146 kDa (see Figure 9B). The introduction of an Nhe I site without additional mutation in pulANheI and the junction of pulA:ces gene fusion were confirmed by DNA sequencing (data not shown).
Figure 7: Outline of *pulA:cex* fusion gene construction. Expression of the fusion gene is controlled by the pUC13 encoded *lac* promoter (single arrowhead) or the subcloned *pulA* promoter (divergent arrowheads). Underlined bases represent codons at the site of gene fusion. The *Nhe* I site in pUC13pulANheI was introduced by PCR modification starting at base pair 2503 in *pulA* (see APPENDIX 7.4). The *Nhe* I site (in the 1458 bp *cex*) is at base pair 127 (see APPENDIX 7.2). The resulting plasmid pUC13pulA:cex also contains the first 176 bp of the 857 bp *pulC* gene. The open rectangles with adjoining arrowheads indicate genes and the direction of transcription, respectively.
pUC13pulA

PCR addition of Nhe I

Nhe I/Hind III
purify 1.7 kb fragment

Nhe I/Hind III
purify 5.7 kb fragment

EcoR I

Hind III

pUC13pulANheI
7.7 kb

pTZEO6
4.5 kb

EcoR I

Hind III

pUC13pulA:ceX
7.4 kb

5' GTC GTA GCT AGC 3'
3.1.3 Construction of the pulA:cenB fusion gene.

The 5551 bp pulA:cenB fusion gene made by fusion of the pulA Nhe I site to the cenB Not I site (Figure 8; see APPENDIX 7.3 and 7.4) is predicted to encode a 1850 amino acid protein with a molecular mass of about 200 kDa (see Figure 9C). The site of gene fusion was sequenced and found to be translationally in-frame (data not shown). This construction encodes the wild type pulA ribosome binding site, translation start codon, the 5' 176 bp of the 857 bp pulC gene as described above for the pulA:cenA (pUC13pulA:cenA) and pulA:ceX fusion genes, and the fusion protein would contain all of the mature protein sequences of CenB.

3.1.4 Construction of a pulA:cenB fusion in the vector pMMB207.

I found, as have others, that plasmids carrying the bla gene (β-lactamase) as the selectable antibiotic resistance marker are not stably maintained in K. oxytoca cells. In my hands fusion protein-encoding plasmids conferring ampicillin resistance (pUC or pTZ-based plasmids) were lost from strains or underwent rearrangements such that they no longer expressed the fusion gene of interest (data not shown). This plasmid loss is believed to be due to a high level of endogenous β-lactamase activity in these cells which allows the survival of plasmid-free cells in the presence of ampicillin (Diethelm et al. 1988). The deleted plasmids are thought to have selective advantage because a high level of fusion protein accumulation is slightly toxic. To circumvent this problem, I switched to the use of the pMMB207 plasmid, which confers chloramphenicol resistance and which exists at a lower copy number than the pUC and pTZ based plasmids (Morales et al. 1991). The plasmid pMMB207 has the additional advantage of encoding the lacIq allele,
Figure 8: Outline of *pulA:cenB* fusion gene construction. Expression of the fusion gene subcloned in pUC13 is controlled by the plasmid encoded *lac* promoter (single arrowhead) or the subcloned *pulA* promoter (divergent arrowheads). Expression of the fusion gene from pMMB207 is controlled by the plasmid encoded *tac* promoter (open arrowhead) and LacI repressor or the subcloned *pulA* promoter. Underlined bases represent codons at the site of gene fusion. The *Nhe* I site in pUC13pulANheI was introduced by PCR modification at base pair 2503 in *pulA* (see APPENDIX 7.4). The *cenB* (3138 bp) *Not* I site starts at base pair 91 (31st codon of the 33 codon signal peptide; see APPENDIX 7.3). The resulting plasmids pUC13pulA:cenB and pMMBpulA:cenB also contain the first 176 bp of the 857 bp *pulC* gene. The open rectangles with adjoining arrowheads indicate genes and the direction of transcription, respectively.
HindIII

pUC13
pulANhel
7.7 kb

NheI

EcoRI

NotI

pTAL3
8.2 kb

HindIII

XbaI

NheI,
Klenow fill-in
HindIII, purify 6 kb
fragment

NoI digest, Klenow fill-in
HindIII, purify 5.3 kb fragment

EcoRI

HindIII

XbaI

pUC13pulA:cenB
11.2 kb

lacIq
Ptac
EcoRI

pMMB207
9.7 kb

CAT

EcoRI

XbaI

EcoRI/XbaI
digest

EcoRI/XbaI
digest

lacIq
Ptac
EcoRI

pMMBpulA:cenB
18 kb

CAT

XbaI

5' GTA GCT AGG GCC 3'
allowing tight IPTG-dependent regulation of cloned genes from the plasmid encoded tac promoter. Transcription of pulA:cenB could be initiated from either the subcloned pulA promoter or the lac (pUC13pulA:cenB) or tac (pMMBpulA:cenB) promoters. This fusion junction was sequenced and found to be translationally in-frame.

3.1.5 Summary of fusion gene constructions.

The four fusion genes engineered and the proteins predicted to result from expression of these constructs in bacterial host cells are shown in Figure 9. All the fusion genes contain approximately the same pulA sequence and most of the individual cellulase encoding genes. In all cases PulA sequences form the amino-terminal portion of each chimera. Fusion between cenA and pulA results in the PulA:CenA chimera containing either 706 (Eco RV) or 847 (Bcl I) amino acids of PulA, approximately 40% of of the CenA CBD, and all of the CenA catalytic domain. PulA:Cex would comprise 834 amino acids of PulA fused to the sequences of mature Cex as the site of fusion was just downstream of the signal peptide cleavage site. Likewise, PulA:CenB would contain 834 amino acids of PulA connected to the mature sequences of CenB.

3.2 Congo red and MUC plate assays of recombinant strains expressing cellulases.

Plate assays of cellulase activity were used as described in MATERIALS AND METHODS (Sections 2.22 and 2.23) to determine if the fusion proteins created retained enzymatic activity.
Figure 9: The fusion *pulA* cellulase genes and corresponding proteins. A: Both *pulA:cenA* fusion genes and resulting PulA:CenA proteins. B: The *pulA:cex* fusion gene and corresponding PulA:Cex chimeric protein. C: The *pulA:cenB* fusion gene and resulting chimeric PulA:CenB protein. The arrowheads represent the subcloned *pulA* promoter. Hatched regions correspond to the *pulA* gene or gene product and stippled regions represent the cellulase portions of each fusion. 1: Catalytic domains. 2: CBD. 3: *Bacillus*-type CBD. 4: Fibronectin-type regions. The heavy black vertical bars represent Pro-Thr rich spacer regions. All the proteins are aligned with the start of the PulA portion of the chimera. The predicted molecular mass of each fusion protein is indicated on the right. The symbol "φ" indicates destroyed restriction sites. The genes are not drawn to scale with their corresponding proteins.
A  
\textit{EcoR V\theta/Pvu II\theta}

\begin{itemize}
  \item PulA:CenA
  \item 124 kDa
\end{itemize}

B  
\textit{Bcl I\theta/BamH I\theta}

\begin{itemize}
  \item PulA:CenA
  \item 140 kDa
\end{itemize}

B  
\textit{Nhe I}

\begin{itemize}
  \item PulA:Cex
  \item 146 kDa
\end{itemize}

C  
\textit{Nhe I\theta/Not I\theta}

\begin{itemize}
  \item PulA:CenB
  \item 200 kDa
\end{itemize}
3.2.1 Cellulase activity associated with *pulA:cenA* expression detected by Congo red plate assay.

When expressed in *E. coli* host cells, both pUC13pulA:cenA and pTZ19pulA:cenA produced enzymes with CMCase activity as determined by Congo red plate assays (Figure 10). The dark circles in panels A and C of Figure 10 are due to cells spotted on the medium surface. Zones of clearing can be seen around spots expressing PulA:CenA (sample 1) and CenA (sample 3) in panels B and D, although the sizes of the cleared zones around the spots of the cells expressing PulA:CenA were smaller than around cells expressing CenA alone. Thus, the CenA segments of these two fusion proteins folded similarly enough to the wild type CenA protein to retain enzymatic activity.

Very small zones of clearing can be seen around cells expressing PulA [negative control; sample 2B: JM101 (pUC13pulA); sample 2D: HB101 (pUC13pulA)]. These are not likely to be the result of PulA activity (PulA hydrolyzes $\alpha$-1,4-linked glycosidic bonds) on CMC (a $\beta$-1,4 linked glucose polymer). These small zones of clearing could be the result of expression of an *E. coli* $\beta$-glycosidase (Al-Zaag 1989). However, this very small clearing was not seen for the control strain in Figure 12 [sample 2: JM83 (pUC13pulA)] and, therefore, is probably a strain-specific phenotype. This question was not investigated further.

The dark areas within the zones of clearing seen in panels B and D are thought to be due to deposition of cell membrane oligosaccharides or proteinaceous materials, remaining after removal of the cells on the medium surface, which stained darkly with Congo red. The intensity of this staining seems to be *E. coli* host strain specific, as
Figure 10: Congo red plate assay of *E. coli* strains expressing the *pulA:cenA* fusion gene. A and B: JM101 (pTZ19pulA:cenA). C and D: HB101 (pUC13pulA:cenA). CMC-containing agar plates with 48 hour bacterial growth before (A and C) and after (B and D) staining with Congo red. 1: PulA:CenA expressing strains. 2: PulA expressing strains. 3: CenA expressing strains. 0.1 mM IPTG and 0.4% maltose were included for the induction of PulA:CenA and PulA, and CenA, respectively. Medium contained 1% CMC.
the CenA expressing strain in panel D was DH5α whereas its neighbour was HB101. Staining differences similar to this have been previously reported (Meinke et al. 1991).

3.2.2 PulA:Cex activity detected by the MUC plate assay.

When *pulA:cex* was expressed from the plasmid pUC13pulA:cex in *E. coli*, a protein was produced that was able to hydrolyse MUC resulting in fluorescence under a UV light source (Figure 11). The cells expressing PulA:Cex (sample 2) clearly fluoresced when illuminated with UV light. However, this fluorescence was not as intense as that produced by the strain expressing wild type Cex (sample 1). The fluorescence of the PulA expressing control strain (sample 3) was not as great as that of either of the strains expressing Cex or PulA:Cex. Thus, the Cex segment of the PulA:Cex fusion protein must have folded in a manner that produced enzymatic activity.

3.2.3 PulA:CenB activity in *E. coli* and *K. oxytoca* host cells detected by the Congo red plate assay.

When the *pulA:cenB* fusion gene was expressed in *E. coli* (pUC13pulA:cenB), an enzyme with CMCase activity was produced, as determined by Congo red plate assays (Figure 12). The clearing produced when *E. coli* (pUC13pulA:cenB) cells were plated (sample 1) was not as large as that produced by *E. coli* (pTAL3) (sample 3), but was clearly larger than the clearing produced by the negative control, the PulA expressing strain (sample 2). As with the CenA and Cex fusion proteins, the CenB segment of the PulA:CenB fusion protein must adopt an overall conformation that allows CenB enzymatic activity. The darkly stained centres of the clearing areas (formerly occupied by cells), again,
Figure 11: Fluorescence of *E. coli* strains growing on TYP medium containing MUC. A: Bacterial growth on medium surface. B: Same plate observed under UV light illumination. 1: Cex expressing strain. 2: PulA:Cex expressing strain. 3: PulA expressing strain (negative control).
Figure 12: Congo red plate assay of *E. coli* strains containing pUC13pulA:cenB. CMC-containing TYP agar plates with 48 hour bacterial growth before (A) and after (B) staining with Congo red. 1: PulA:CenB expressing strain. 2: PulA expressing strain (negative control). 3: CenB expressing strain.
could be the result of oligosaccharides or other materials left by cells on the medium surface, which stained intensely with Congo red. Strains 1 and 2 are in *E. coli* C600 whereas 3 is in HB101 and this may contribute to the differences in observed spot intensities. (However, the HB101 strain in Figure 10 did not stain in this manner.)

Expression of *pulA:cenB* from pMMBpulA:cenB maintained in *K. oxytoca* cells resulted in expression of a protein with CMCase activity (Figure 13). The clearing produced by cells of *K. oxytoca* (pMMBpulA:cenB) (sample 1) spotted onto CMC-containing plates appears as large as that produced by *K. oxytoca* (pMMBcenB) (sample 3) and was clearly larger than the clearing produced by the negative control (pMMB207 containing strain; sample 2). These data show that the CenB segment of the PulA:CenB fusion protein from pMMBpulA:cenB must adopt an overall conformation such that enzymatic activity is retained in *K. oxytoca* cells. The darkly stained centres of the clearing (area formerly occupied by cell spots), again, could be the result of membrane oligosaccharide or other materials left by cells on the medium surface, which stained intensely with Congo red. The vector control, *K. oxytoca* (pMMB207), did not produce a zone of clearing on the Congo red plate assay. Therefore, unlike the situation for *E. coli*, *K. oxytoca* does not seem to produce a β-1,4 glycosidase that hydrolyses CMC (see Section 3.2.1 and Figure 10).

### 3.3 Induction and kinetics of expression of PulA:CenB.

#### 3.3.1 Determination of optimal IPTG concentration for induction of *pulA:cenB*.

The induction of expression of PulA:CenB from
Figure 13: Congo red plate assay of *K. oxytoca* (pMMBpulA:cenB). CMC-containing agar plates with 48 hour bacterial growth before (A) and after (B) staining with Congo red. 1: PulA:CenB expressing strain. 2: pMMB207 containing strain (negative control). 3: CenB expressing strain.
pMMBpulA:cenB by 0.01, 0.1, 0.17, 0.25, 0.33, 0.4, and 0.5 mM IPTG was assessed. Cultures of *K. oxytoca* (pMMBpulA:cenB) were grown in TYP liquid medium to which was added the appropriate IPTG concentration at mid-logarithmic growth phase. The cultures were incubated for a further 5 hours before the cells were separated from the medium by centrifugation. Culture fractions (cell-free medium and cells) were analysed by Western blot using anti-CenB antiserum (Figure 14; for expression of PulA:CenB in the absence of IPTG see Figure 15, 0 hour lane). The optimal IPTG concentration for high level of induction was observed to be 0.1 mM (maximum accumulation of PulA:CenB after 5 hours). Consequently, 0.1 mM IPTG was used in all subsequent induction experiments. Several bands corresponding to fusion protein presumed proteolytic products were detected (discussed below), as well as a 200 kDa band that was thought to be the full-length PulA:CenB protein (indicated by the arrowhead). None of these IPTG concentrations resulted in detectable secreted PulA:CenB on the basis of this analysis of cell-free culture supernatant fluids.

3.3.2 Kinetics of PulA:CenB accumulation.

The IPTG (0.1 mM) induction of pulA:cenB expression was monitored over a 24 hour period by Western blot analysis (Figure 15). Cells of *K. oxytoca* (pMMBpulA:cenB) showed a detectable amount of the fusion protein along with proteins of approximately 118 and 47 kDa after 0.5 hours of induction. The amounts of the full-length protein and proteolytic species increased over time, reaching a maximum concentration after 5 hours of induction. Along with the appearance of PulA:CenB, the concomitant appearance of the two smaller bands (118 and 47 kDa species) could be due to a precursor-product relationship between the full-length and smaller bands, suggestive of proteolysis.
Figure 14: Western blot of an IPTG titration of a *K. oxytoca* (pMMBpulA:cenB) culture. *K. oxytoca* (pMMBpulA:cenB) was grown to mid-log phase, IPTG added, and induction continued for 5 hours. Each lane was loaded with an equal number of cells (or equivalent volume of cell-free medium). C: Cells. M: cell-free culture medium. Molecular mass markers (kDa) are indicated on the left. Arrowhead indicates full-length PulA:CenB protein migration. (see Figure 15 for data which shows absence of CenB immunoreactive species without induction.)
Figure 15: Western blot of an IPTG induction time course of a *K. oxytoca* (pMMBPulA:cenB) culture. *K. oxytoca* (pMMBPulA:cenB) was grown to mid-log phase, 0.1 mM IPTG added, and samples taken at the times indicated. Each lane was loaded with an equal number of cells (or an equivalent volume of cell-free medium). C: cells. M: cell-free culture medium. Molecular mass markers (kDa) are indicated on the left. Arrowhead indicates migration of full-length PulA:CenB.
The accumulation of the full-length PulA:CenB, the 118 kDa, and the 47 kDa species decreased by 22 hours after induction to the level seen at 1 hour post induction. The dramatic decrease in intensity of the full-length band in the culture medium between the 5 and 22 hour time points (relative to the 118 and 47 kDa species) indicates that proteolysis occurred in the culture medium as well as within cells, and supports the interpretation that degradation of the full-length protein gives rise to the smaller species.

After 4 hours of IPTG induction, the full-length PulA:CenB, 118 kDa, and 47 kDa species appeared in the culture medium. Since maltose was not included in the growth medium it is not likely that the proteins were secreted via the PulA-specific pathway (for analysis of PulA secretion from *K. oxytoca* see Section 3.8). Although it is possible that these proteins appear in the culture medium because of cell lysis or non-specific leakage through the cell envelope, this was not directly tested in this experiment [however, cell lysis was observed when intact cells were assayed for CMCase activity (see Section 3.5.1)].

The band co-migrating with the 118 kDa protein marker is hereafter referred to as the 100 kDa protein species, and the second fragment of interest co-migrating with the 47 kDa protein marker is referred to as the 47 kDa species. Since these polypeptides were observed in samples prepared by boiling intact cells in SDS-PAGE loading buffer immediately after sampling, I suspect that proteolysis occurred during culture growth as opposed to after harvest. Previous studies of CenB expressed in *E. coli* or *C. fimi* identified a 47 kDa protein species as being the result of proteolysis occurring between the *Bacillus*-type CBD and the amino-proximal fibronectin-type region (Meinke *et al.* 1992 and Sandercock *et al.* 1996), and amino acid sequencing of the N-terminal
end of the *E. coli* 47 kDa species indicated that proteolysis occurred at two closely spaced sites (Meinke *et al.* 1992).

3.4 Quantification of PulA:CenB and CenB accumulation in *K. oxytoca* cells.

Using the results of the induction experiment shown in Figure 15, I determined the maximum accumulation of PulA:CenB and CenB in cultures of *K. oxytoca* (pMMBpulA:cenB) and *K. oxytoca* (pMMBcenB), respectively, by semi-quantitative Western blotting. Cultures grown in TYP liquid medium were induced with 0.1 mM IPTG for 5 hours. Cell extracts of these cultures in parallel with known amounts of purified CenB were subjected to SDS-PAGE followed by Western blotting with anti-CenB antibodies (Figure 16). Densitometry of the Western blot shown in Figure 16 indicated that ca. 17 mg/L of the presumed full-length PulA:CenB protein was produced (and retained) by *K. oxytoca* (pMMBpulA:cenB) cells after a 5 hour induction with 0.1 mM IPTG (200 kDa band in lane 4). In contrast, at least 67 mg/L of full-length CenB was accumulated by cells of *K. oxytoca* (pMMBcenB) cultures grown under similar conditions of induction (100 kDa band in lane 2). However, in both strains large amounts of presumed degradation products were also seen, which indicates an underestimation of the total amount of full-length protein synthesized.

These data were difficult to interpret since the amount of detected CenB from *K. oxytoca* (pMMBcenB) was above the range of purified CenB used, and only the lowest dilution of *K. oxytoca* (pMMBpulA:cenB) extract tested was within this range. However, the measured intensities of the bands in lanes 1 and 2 and lanes 3 and 4 were proportional to their relative concentration. Therefore, since these intensities were within the linear range of the scanning densitometer,
Figure 16: Quantitative Western blot of *K. oxytoca* (pMMBcenB) and *K. oxytoca* (pMMBpulA:cenB) cell extracts to determine the approximate amounts of CenB and PulA:CenB produced. Lanes 1, 2: *K. oxytoca* (pMMBcenB). Lanes 3, 4: *K. oxytoca* (pMMBpulA:cenB). Lanes 5 (50 ng), 6 (25 ng), and 7 (10 ng): Purified CenB. Dilutions made: 0.1 (lanes 1, 3), and 0.05 (lanes 2, 4). The arrowhead indicates the migration of PulA:CenB. Molecular mass markers (kDa) are indicated on the left.
these data were used while noting that the amount of CenB produced is likely to be greater than this estimate.

Under these conditions ca. 4 times more (on a mass basis) of full-length CenB than full-length PulA:CenB accumulated in cells and very little was secreted to the extracellular medium (see Figure 15). It is possible that these quantitative methods are not very reliable as the CenB antibodies may interact with different affinities to CenB and the full-length PulA:CenB fusion protein. However, these experiments imply that approximately 0.015 pg/cell (about 45 000 molecules) of the PulA:CenB fusion protein was synthesized.

3.5 Analyses of CMCase activity in strains expressing PulA:CenB.

The Congo red plate assays indicated that K. oxytoca (pMMBpulA:cenB) cells produced an enzyme with CMCase activity (see Figure 13). The Western blot analysis gave an approximate amount of the fusion protein made, and indicated that there was proteolytic degradation of the full-length fusion protein into a few prominent cleavage products. However, neither the enzymatic activity of the various species was known nor where in the cell they were localized. Therefore, in order to obtain quantitative CMCase activities and to monitor the distribution of activity within various culture compartments, CMCase activity was assayed using intact cells (cell-surface associated activity), cell lysates (total cell-associated activity), and cell-free supernatant fluids of induced cultures. Cultures of K. oxytoca (pMMBpulA:cenB) and K. oxytoca (pMMBcenB) were grown in TYP liquid medium using two different growth conditions: either with or without maltose supplementation for the induction of the PulA-specific secretory proteins. Both types of cultures were induced for 5 hours with 0.1 mM IPTG, after
which the various fractions were collected and CMCase activity measured using the DNS assay (Miller 1959) for rates of reducing sugar production (Table III).

In the presence or absence of maltose ca. 80% of the total CMCase activity associated with *K. oxytoca* (pMMBpulA:cenB) cultures remained intracellular (cell lysates), with 18% exposed at the cell surface and ca. 2% localized in the cell-free culture medium. Interestingly, the data from *K. oxytoca* (pMMBcenB) indicated that ca. 40% of the CMCase activity was cell-surface exposed (44% IPTG-induced and 39% IPTG + maltose-induced). This distribution is similar to that of PulA activity in *K. oxytoca* (pMMB207) cells in which ca. 40% of PulA activity was localized at the cell surface (see Section 3.8). Since CenB would not be predicted to be anchored to the outer membrane and Western blot analysis of the cell-free culture media failed to detect any protein, it was surprising to detect enzymatic activity associated with the outer surface of *K. oxytoca* (pMMBcenB) cells. However, it was possible that CenB polypeptides from these *K. oxytoca* (pMMBcenB) cells were exposed to the extracellular medium via an alternative pathway. Indeed, some CenB proteins were detected in Western blots of outer membrane preparations of strains *K. oxytoca* (pMMBcenB) (see Section 3.7). However, since most of the CenB protein detected in these cells was in the form of inclusion bodies (some of which co-purified with the periplasmic fraction; see Section 3.7), it was possible that the accumulation of inclusion bodies in *K. oxytoca* (pMMBcenB) disrupted cell integrity in the assay medium such that intracellular CenB was released during the enzyme assay, resulting in high CMCase activity associated with "intact" cells.
Table III: Endoglucanase activity of cultures grown in TYP medium.

<table>
<thead>
<tr>
<th>Strain</th>
<th>0.1 mM IPTG</th>
<th>0.1 mM IPTG + 0.4% Maltose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell’s surface</td>
<td>Cell lysate</td>
</tr>
<tr>
<td><em>K. oxytoca</em> (pMMBpulA:cenB)*2</td>
<td>17</td>
<td>81</td>
</tr>
<tr>
<td><em>K. oxytoca</em> (pMMBcenB)*3</td>
<td>44</td>
<td>52</td>
</tr>
</tbody>
</table>

1Expressed as a % of the total CMCase activity.

2Total activity: 36 (IPTG) or 23 (IPTG + maltose) μmoles glucose equivalents released/minute/ml.

3Total activity: 314 (IPTG) or 191 (IPTG + maltose) μmoles glucose equivalents released/minute/ml.
3.5.1 Lysis and/or outer membrane disruption of cells assayed for CMCase activity.

The high level of apparent surface exposure of CMCase activity associated with *K. oxytoca* (pMMBcenB) cultures in contrast to the low cell surface CMCase activity associated with *K. oxytoca* (pMMBpulA:cenB) cultures suggested that either CenB was anchored to the outer membrane or that membrane integrity of *K. oxytoca* (pMMBcenB) cells was disrupted in the assay mixture. To test for membrane disruption, aliquots from the CMCase assay mixture were removed after a 30 minute (at 37° C) incubation period, but before the DNS reagent was added; cells were removed from the solution by centrifugation, and the cell-free CMCase assay mixture assayed for β-galactosidase (indicative of cell lysis or at least leakage of cytoplasmic contents) and PhoA (indicative of outer membrane disruption) activity. β-galactosidase and PhoA activity in CMCase assay mixtures of intact cell samples were compared to French pressed extracts of *K. oxytoca* (pMMB207), *K. oxytoca* (pMMBcenB), and *K. oxytoca* (pMMBpulA:cenB) and are summarized in Table IV.

The amount of β-galactosidase activity released from intact cells of the strain *K. oxytoca* (pMMB207) was < 1.0 Miller units (IPTG-induced and IPTG + maltose-induced). This is less than 0.5% of the intracellular β-galactosidase activity measured in French pressed samples. Significantly higher β-galactosidase activity was released from intact cell samples of *K. oxytoca* (pMMBcenB) cultures (157.5 and 112.4 Miller units in IPTG and IPTG + maltose-induction, respectively). These results indicate that ca. 34% of the intracellular β-galactosidase activity of cells induced with IPTG and ca. 74% of the activity of IPTG + maltose-induced cells was released during incubation with the CMCase substrate.
Table IV: β-galactosidase and alkaline phosphatase (PhoA) activity released from cells in the CMCase assay.

<table>
<thead>
<tr>
<th>Strain</th>
<th>0.1 mM IPTG</th>
<th>0.1 mM IPTG + 0.4% Maltose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intact cells</td>
<td>Lysate</td>
</tr>
<tr>
<td><em>K. oxytoca</em> (pMMB207)</td>
<td>&lt; 1.0</td>
<td>559.4</td>
</tr>
<tr>
<td><em>K. oxytoca</em> (pMMBpulA:cenB)</td>
<td>8.4</td>
<td>435.5</td>
</tr>
<tr>
<td><em>K. oxytoca</em> (pMMBcenB)</td>
<td>157.5</td>
<td>459.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strain</th>
<th>0.1 mM IPTG</th>
<th>0.1 mM IPTG + 0.4% Maltose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intact cells</td>
<td>Lysate</td>
</tr>
<tr>
<td><em>K. oxytoca</em> (pMMB207)</td>
<td>&lt; 1.0</td>
<td>52.7</td>
</tr>
<tr>
<td><em>K. oxytoca</em> (pMMBpulA:cenB)</td>
<td>9.6</td>
<td>55.3</td>
</tr>
<tr>
<td><em>K. oxytoca</em> (pMMBcenB)</td>
<td>16.0</td>
<td>21.8</td>
</tr>
</tbody>
</table>

1 Activity expressed as Miller units. Intact cells: activity released from intact cells. Lysate: activity released by French press treatment.
2 Cells/Lysate: % activity released from intact cells relative to activity released by French press treatment.
(4% CMC). In contrast, \textit{K. oxytoca} (pMMBpulA:cenB) intact cells released less than 8% of the intracellular $\beta$-galactosidase activity (either for IPTG or IPTG + maltose induction). Therefore, intact cells of \textit{K. oxytoca} (pMMBcenB) appeared to lyse in the CMCase assay reagents whereas cells of \textit{K. oxytoca} (pMMBpulA:cenB) remained mostly intact.

The amount of PhoA activity released from \textit{K. oxytoca} (pMMBcenB) cells was greater (73% for IPTG-induced and 100% for IPTG + maltose-induced) than for \textit{K. oxytoca} (pMMBpulA:cenB) cells (17% for IPTG-induced and 60% for IPTG + maltose-induced). In contrast, the outer membrane of cells of the \textit{K. oxytoca} (pMMB207) control strain released < 3% of the intracellular PhoA activity from intact cells grown under either condition. Therefore, these data indicate that intact cells of both \textit{K. oxytoca} (pMMBpulA:cenB) and \textit{K. oxytoca} (pMMBcenB) lost outer membrane integrity in the CMCase assay.

These results suggest that the outer membrane of cells expressing PulA:CenB or CenB are disrupted whereas strains expressing CenB appeared to lyse in the CMCase assay. The assay components (50 mM KPO$_4$ (pH 7.0) buffer and 4% CMC) may be responsible for lysis or outer membrane disruption. However, since \textit{K. oxytoca} (pMMB207) cells maintained their integrity in the CMCase assay, it is likely that PulA:CenB and CenB expression results in \textit{K. oxytoca} cells that suffer outer membrane disruption and/or are lysed (in the case of CenB expression) in the assay reagents, releasing their periplasmic and/or cytoplasmic contents. In light of these results, the CMCase assay data (Section 3.5) must be re-interpreted as indicating that a significant portion of the CMCase activity measured from supposedly intact cell samples was the result of intracellular enzymes released into the assay mixture. Therefore, CenB-expressing strains do not secrete significantly
more protein to the cell surface than PulA:CenB expressing strains, and certainly not more than the 40% of PulA secreted to the cell surface of wild type cells (see Section 3.8).

3.6 Analyses of PulA:CenB presumed proteolytic products.

Expression of PulA:CenB resulted in the production of a number of anti-CenB antibody immunoreactive species detected in Western blots, more than one of which could potentially have retained CMCase activity. Furthermore, the kinetics of accumulation and disappearance of these species indicated that the full-length protein was made and subsequently degraded. Since the 100 and 47 kDa presumed proteolytic species both bound to avicel, as did the full-length PulA:CenB, all these molecules contained a CBD (Figure 17). Therefore, it was of interest to determine whether PulA:CenB and/or the 100 kDa proteolytic product had CMCase activity (published results have indicated that a 47 kDa species produced in \textit{E. coli} does not have CMCase activity; Meinke \textit{et al.} 1991). To answer this question two approaches were used: 1) zymogram analysis: and 2) CMCase assays on the full-length 200 kDa and 100 kDa fragment partially separated from each other on the basis of molecular mass by ultrafiltration through appropriate molecular mass cut-off membranes.

3.6.1 Zymogram analysis.

Zymograms were initially prepared using the CMC overlay method described in Section 2.14. To simplify the zymogram assay and reduce the potential for protein diffusion during contact time between the polyacrylamide gel and the agarose overlay, I developed a new zymogram method that included 0.01% CMC in the polyacrylamide gel matrix. Protein samples were prepared as described in Section 2.14.
Figure 17: Western blot of avicel-bound extracts of *K. oxytoca* (pMMBpulA:cenB). 1: Untreated cell extract. 2: avicel bound extract. The arrowhead indicates the migration of PulA:CenB. Molecular mass markers (kDa) are indicated on the left. Proteins were detected with anti-CenB antibodies.
Following electrophoresis, proteins in these gels were renatured by incubating in 5 volumes 50 mM KPO$_4$ (pH 6.9; 4 X 30 minutes), the gel incubated overnight at 30° C, and CMC hydrolysis visualized by staining with Congo red and then destaining as described in Section 2.14.

The Congo red staining, followed by Coomassie staining, of an initial polyacrylamide gel prepared with CMC in the separating matrix revealed a faint clear zone close to the 199 kDa marker, presumably PulA:CenB (Figure 18A, lane 1). However, the predominant regions of clearing were in the range of the 106 and the 69 kDa protein markers. This zymogram pattern is similar to that produced by CenB from *K. oxytoca* (pMMBcenB) culture extracts (Figure 18A lane 2). These data also show very faint clearings around the 199 kDa and below the 44 kDa molecular mass markers (lane 2). Resolution of the clearing zones produced by protein species between 200 and 69 kDa was not very good, therefore longer gels were run (Figure 18B and C). The multiple zones of clearing seen between the 118 and 69 kDa protein markers (for both PulA:CenB and CenB samples) in these zymograms are consistent with the pattern seen in published zymograms of extracts of *E. coli* strains expressing CenB, done with the overlay method (Owolabi et al. 1988). The multiple zones of clearing seen indicate that there are many different sites of proteolysis resulting in a wide variety of protein species containing the CenB catalytic domain. Since proteolytic products corresponding to all clearings were not seen, it is likely that the anti-CenB antibodies used reacted with different affinities to the different protein species. However, these data support the idea that the full-length fusion protein was cleaved at or near the PulA:CenB fusion junction, resulting in more-or-less complete release of the 100 kDa CenB portion of the chimeric protein. At this point however, I am unable to rule out the possibility of more complex patterns of proteolysis resulting in a
Figure 18: Identification of enzymatically active protein species by zymogram. A: Congo red and Coomassie stained polyacrylamide gel containing 0.01% CMC. B: Congo red stained zymogram. C: Western blot using anti-CenB polyclonal antibodies. 1: *K. oxytoca* (pMMBpulA:cenB) French pressed, cleared extract. 2: *K. oxytoca* (pMMBcenB) French pressed, cleared extract. Molecular mass markers (kDa) are indicated on the left. Arrowhead indicates full-length PulA:CenB protein migration.
100 kDa species that retains CMCase activity. Amino-terminal protein sequencing could in principle be used to determine the identity of the 100 kDa species (see Section 3.6.4).

It was possible that the presence of 0.01% CMC in the gel matrix might have affected protein migration either because of CMC binding by the CBD domains of the proteins or by decreasing the gel pore size. However, a comparison of the migration of the protein species of interest in gels containing 0.01% CMC (Figure 18C) with protein migration in gels lacking CMC (Figure 14) indicated that there was no noticeable effect on migration relative to the molecular mass standards.

Although Western blot analysis of these samples showed amounts of full-length PulA:CenB similar to the 100 kDa protein species (Figure 18C), the enzyme activity associated with the full-length protein produced a much smaller zone of clearing in the zymogram than did the 100 kDa protein species (Figure 18B). Scanning densitometry was used to measure the areas of clearing produced by different amounts of the various protein species on a zymogram (Figure 18B shows 1 of the 4 lanes scanned). In this way I was able to estimate that the full-length protein produced a clearing which was at most 17% the size of the 100 kDa protein species. However, this is only an upper-end estimate as the clearing due to the 100 kDa protein species was still saturating when the clearing at 200 kDa was no longer visible on the zymogram.

3.6.2 Analyses of denatured and native protein electrophoretic migration.

The zymograms described above were prepared using protein samples that were not denatured by boiling in SDS-PAGE buffer
containing BME (see below). Therefore, I was concerned that the migration of native proteins would be different from that of their fully denatured forms. Using Western blot analysis to compare the migration of immunoreactive species in the BME/boiled samples (standard SDS-PAGE prepared samples) to the migration of samples that were prepared in loading buffer lacking BME at room temperature (the procedure used for zymograms) I was able to determine which species created the clearings seen on zymograms. The migration of both CenB and PulA:CenB species was not greatly affected by boiling in loading buffer containing BME (Figure 19). Therefore, the different cleared zones in the zymograms are likely to be the result of the different corresponding presumed proteolytic products seen in the conventional Laemmli-gel Western blots. Although equal amounts of denatured and native protein samples (based on volume of cell extract) were loaded into the wells of the SDS gel used for this Western blot, the intensity of the undenatured protein was higher than that of the denatured sample. This difference in intensities seen between denatured and native samples is likely to be due to differences in antibody-epitope recognition between the denatured or native protein conformations by the anti-CenB polyclonal antiserum. Alternatively, these might indicate binding of the various protein species to each other during electrophoresis in the native gel system. However, since the intensity of both the 200 and 100 kDa protein species decreased in the denatured sample compared to the undenatured sample with no significant appearance of additional bands, it seems likely that there were fewer epitopes available for CenB antibody recognition in the denatured samples. Since native CenB was used to prepare the anti-CenB antibodies (E. Kwan, personal communication), the most likely explanation is that the non-denatured PulA:CenB polypeptide presents more epitopes to the CenB antibodies.
Figure 19: Comparison of electrophoretic mobility of native and denatured PulA:CenB by Western blot. 1 and 2: Native extracts. 3 and 4: denatured extracts. Lanes 1 and 3: 15 μL cell extract. Lanes 2 and 4: 10 μL cell extract. The arrowhead indicates the migration of full-length PulA:CenB. Molecular mass markers (kDa) are indicated on the left.
3.6.3 Partial purification of the 100 and 200 kDa species.

As noted above, the Western blot analyses of non-denatured protein species indicated that similar amounts of full-length PulA:CenB and the 100 kDa proteolytic product (possibly the complete CenB protein) were present in the zymogram assays (based on intensity of immunoreactive species). However, the clearing produced by PulA:CenB was determined to be at most 17% the size of the clearing produced by the 100 kDa proteolytic product. This difference in clearing size may have been a reflection of a lower specific activity of the full-length fusion protein, perhaps because the PulA part of the protein interfered with the catalytic activity of the CenB segment. Alternatively, the larger PulA:CenB protein may renature less efficiently in the polyacrylamide gel matrix, resulting in the lower activities in the zymogram. To differentiate between these two possibilities, ultrafiltration was used to obtain fractions of *K. oxytoca* (pMMBpulA:cenB) cells enriched for the full-length 200 kDa protein species or the 100 kDa proteolytic species (complete separation was not possible using this technique; Figure 20A), and CMCase activity in these fractions was measured (Figure 20B). The Western blot data indicate that there were similar amounts of the 100 kDa proteolytic species in both fractions (>100 and <100 kDa; in this gel two bands migrating between the 83 and 175 kDa protein markers were resolved). Since previous zymograms indicated that the 47 kDa species had very little CMCase activity [previously published data also indicated that a 47 kDa species lacks activity (Meinke *et al.* 1991)], therefore, the difference in CMCase activity between the two fractions (35 X 10^-2 - 28 X 10^-2 = 7 X 10^-2 μmoles glucose released/min/ml) was used as an estimate of activity attributable to full-length PulA:CenB.

Densitometric analysis of the blot in Figure 20A indicated that
Figure 20: Estimation of PulA:CenB enzymatic activity. A: Western blot showing enrichment of the different protein species probed with an anti-CenB polyclonal antibody. Arrowhead indicates full-length PulA:CenB protein migration. Molecular mass markers (kDa) are indicated on the left. B: CMCase activity of each size-enriched fraction. 1X 10^-2 μmoles glucose released/min./ml. Numbers within parentheses are standard deviations of three assays.
there was ca. 17 times more protein in the bands migrating with the 100 kDa protein marker than in the band migrating with the 200 kDa protein marker (completely denatured samples). Therefore, setting the relative amount of full-length protein at 1 and the amount of the 100 kDa species at 17, I was able to estimate (normalized) relative activities, from which I concluded that the full-length PulA:CenB chimera had approximately 4 times higher relative activity (7 X 10^{-2} μmoles glucose released/min/relative protein content) than the activity associated with the 100 kDa protein species (1.6 X 10^{-2} μmoles glucose released/min/relative protein content). In contrast, the zymogram shown in Figure 18 indicated that the 200 kDa protein species had ca. 17% (1/6th) the activity of the 100 kDa protein species. Therefore from this preliminary comparison, I conclude that enzymatic activity of the the full-length PulA:CenB protein lies somewhere between 4 times more and 6 times less than the CMCase activity measured for CenB; namely, these two proteins have approximately the same specific activities.

3.6.4 N-terminal amino acid sequencing.

The 100 kDa protein species may be the product of a single cleavage at the fusion joint between the PulA and CenB segments of the PulA:CenB protein, or it may contain portions of both segments as a result of more complex proteolysis. Therefore, N-terminal sequencing by Edman degradation was attempted to determine the identity of the 100 kDa species. The ambiguous N-terminal sequencing results of the avicel affinity-enriched species, separated by SDS-PAGE, and N-terminal de-blocked prior to sequencing suggested that the 100 kDa protein species is a mixture of two or more polypeptides (data not shown; the resolution of protein species on the gels used for N-terminal sequencing were similar to that of Figure 20A in which two minor species bracket one intense
To determine whether the difficulty in obtaining N-terminal sequence data was due to close migration of similarly sized proteolytic products of PulA:CenB in one dimensional SDS-PAGE, two dimensional electrophoresis was used. Polypeptides separated by isoelectric focusing and then by molecular mass (SDS-PAGE) were detected by Western blot using anti-CenB antibodies. One such experiment is shown in Figure 21. The three major and two minor protein spots at arrowhead "b" (and the two others just below it) indicated that there were several similarly sized protein species, confirming my suspicion that the protein band resolved in one dimensional SDS-PAGE was indeed a mixture of at least two different species. At least one of these species has CMCase activity (see the zymogram in Figure 18) and therefore contains the CenB catalytic domain.

Assuming that the 47 kDa species (arrowhead “c” in Figure 21) consisted of CenB sequences extending from the amino-proximal fibronectin region to the C-terminus (see Figure 22), it was predicted to have an isoelectric point (pI) of 4.24. A 100 kDa CenB species (arrowhead b) extending from the fusion junction to the C-terminus was predicted to have a pI of 4.79. The full-length PulA:CenB (arrowhead “a”) would have a pI of 4.54 [these values were calculated from the deduced protein sequences using the algorithms available with the GeneWorks 2.45 software package (IntelliGenetics, Inc.)]. Based on the migration of internal protein pH markers, the 47 kDa and 100 kDa species migrated to isoelectric points consistent with the above assumptions. However, the 200 kDa species had an experimentally determined pI of 4.79, instead of the calculated value of 4.54. As previously described (see Section 1.5.1), the native PulA protein is post-translationally modified by the addition of a glycerol molecule with two
Figure 21: Western blot of a 2-D gel of one *K. oxytoca* (pMMBpulA:cenB) culture extract. The isoelectric focusing occurred from right (basic end) to left (acidic end). The SDS-PAGE ran from top to bottom. Arrowheads a, b, and c, correspond to the migration of the full-length PulA:CenB, 100 kDa proteolytic species, and the 47 kDa proteolytic species, respectively. Molecular mass markers (kDa) are indicated on the left.
Acidic

Basic

\[ \text{a} \rightarrow \]

\[ \text{b} \rightarrow \]

\[ \text{c} \rightarrow \]
attached fatty acids to the sulfhydryl group of the amino-terminal cysteine as well as by the addition of a third fatty acid to the amino group of this same amino-terminal cysteine (Pugsley et al. 1986). Therefore, this difference in migration in the 2-D gel system could be a result of modification of PulA:CenB. Since the effects of fatty acid modification on protein migration in isoelectric focusing must be determined empirically (by analysing the migration in 2-D gels of PulA:CenB +/- fatty acid modification) and these experiments have not been done, I cannot confirm this hypothesis.

Preparative 2-D gel electrophoresis was attempted to separate the two 100 kDa protein species in sufficient quantity for N-terminal sequencing. However, perhaps due to the acidic nature of these polypeptides, the proteins precipitated in the basic end of the IEF gel (high concentrations of acidic proteins are prone to precipitation in the 2-D gel loading buffer; Investigator™ 2-D electrophoresis system manual, Millipore) and these attempts were not successful (data not shown).

It seems clear that proteolysis of PulA:CenB occurs in vivo, since degradation products were observed in samples prepared by boiling intact cells in SDS-PAGE loading buffer immediately after harvesting. It also seems from N-terminal sequencing that proteolysis does not occur at single sites to provide single fragments that correspond to the individual bands seen in SDS-PAGE. Proteolysis may occur at more than one site within a small region, generating similarly sized proteins with slightly different N-termini, or proteolysis may occur at distant sites generating fragments of similar size that remain immunoreactive to anti-CenB antibodies and bind to avicel. Considering the sizes of the major proteolytic products (100 and 47 kDa), their immunoreactivity to CenB antibodies, their ability to bind to avicel, and the 2-D Western blotting
results, the simplest interpretation is that there is proteolysis of PulA:CenB in the C-terminal region of the CenB segment, resulting in the 47 kDa species, and at more than one closely-spaced site at the junction of the protein fusion, resulting in the release of more-or-less native CenB. Thus I propose that proteolysis occurs in the linker region connecting the amino-proximal *Bacillus*-type CBD to the fibronectin region, and between the two halves of the fusion protein, resulting in accumulation of the 100 and 47 kDa species (Figure 22). Therefore, the 100 kDa proteolytic species would correspond approximately to the entire CenB portion of the chimera whereas the 47 kDa fragment would correspond to the C-terminal CBD of CenB. The 160 and 60 kDa fragments concomitantly produced could correspond to one of the minor species detected between the 200 and 118 kDa and between the 78 and 47 kDa protein markers (see Figure 15).

3.7 Sub-cellular distribution of PulA:CenB.

Western blot analysis (Figure 15) indicated that very little PulA:CenB was secreted from *K. oxytoca* (pMMBpulAxenB) cells to the extracellular TYP medium. The CMCase activity data together with the lysis data (Tables III and IV, respectively) indicated that very little CMCase activity was detected on the surface of cells, and so support this conclusion. Therefore, in order to obtain some understanding of the bottleneck of PulA:CenB secretion from cells of *K. oxytoca*, I wished to determine the site(s) of accumulation of PulA:CenB within cells of *K. oxytoca* (pMMBpulAxenB).

Cells expressing PulA:CenB were grown in TYP medium +/-maltose (maltose supplemented cultures were used to see if PulA:CenB accumulated in different compartments as a result of expression of the PulA-specific secretory proteins). The cells concentrated from these
Figure 22: Diagrammatic representation of PulA:CenB with potential sites of proteolysis and resulting protein fragments. 1: PulA portion of the chimera. 2 - 5: CenB portion of the chimera. 2: CenB catalytic domain. 3: Bacillus-type CBD. 4: fibronectin-type regions. 5: C. fimi-type CBD. Arrowheads indicate potential sites of proteolysis in K. oxytoca. Heavy horizontal bars, with corresponding molecular masses, represent potential CenB-immunoreactive proteolytic products. The CenB domain structure is modified from Meinke et al. 1992.
cultures were split into two portions. One portion was kept for analysis of total CenB-immunoreactive species (referred to as intact cells) while the second portion was fractionated into sub-cellular compartments. The periplasmic proteins of such cultures were enriched by osmotic shock as described in MATERIALS AND METHODS (Section 2.15). The resultant sphaeroplasts were disrupted by French press and the cytoplasmic proteins obtained by collection of the supernatant liquids after ultracentrifugation. The pellet resulting from ultracentrifugation contained both the inner and outer membranes, which were resuspended and separated from each other by sucrose density gradient centrifugation. The distribution of full-length PulA:CenB and proteolytic products in all of these fractions was evaluated by Western blot analysis in comparison to intact cells.

The efficiency of fractionation was monitored by assay of β-galactosidase (representative of the cytoplasmic fraction), alkaline phosphatase (representative of the periplasmic fraction), and NADH dehydrogenase (representative of the inner membrane fraction) activities. The outer membrane fraction was identified by its position in sucrose density gradients and confirmed by 2-keto-3-deoxyoctonate (KDO) analysis (Table V). Each fraction had the highest measured level for its particular marker; i.e., the cytoplasmic fraction had the highest β-galactosidase activity, the periplasmic fraction had the highest alkaline-phosphatase activity, and so on. Therefore, the methods used for cell fractionation provided a good separation of these cellular compartments.

In Western blots, full-length PulA:CenB was found in all the fractions, including the inner and outer membranes, with the exception of the periplasm, when cultures were supplemented with maltose (Figure 23). The presence of PulA:CenB associated with the inner membranes of
Table V: Enzyme activities\(^1\) and KDO contents of a representative fractionation of a *K. oxytoca* (pMMBpulA:cenB) culture grown in TYP medium.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>(\beta)-galactosidase(^2)</th>
<th>Alkaline phosphatase(^3)</th>
<th>NADH dehydrogenase(^4)</th>
<th>KDO(^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free culture</td>
<td>1</td>
<td>0.1</td>
<td>&lt; 0.6</td>
<td>4</td>
</tr>
<tr>
<td>supernatant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact</td>
<td>9</td>
<td>5</td>
<td>26</td>
<td>4</td>
</tr>
<tr>
<td>cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>62</td>
<td>4</td>
<td>2</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Periplasm</td>
<td>23</td>
<td>90</td>
<td>0.6</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Inner membrane</td>
<td>5</td>
<td>0.8</td>
<td>70</td>
<td>13</td>
</tr>
<tr>
<td>Outer membrane</td>
<td>&lt; 0.1</td>
<td>0.1</td>
<td>0.7</td>
<td>78</td>
</tr>
</tbody>
</table>

\(^1\) Activities are expressed as a percentage of the totals.
\(^2\) Total activity: 150.0 Miller units/mg total protein.
\(^3\) Total activity: 507.7 Miller units/mg total protein.
\(^4\) Total activity: 4.5 \(\Delta\text{OD}/\text{min/mg total protein.}\)
\(^5\) Total activity: 4.9 mg KDO/mg total protein.
Figure 23: Sub-cellular distribution of PulA:CenB and CenB. 1 - 11: *K. oxytoca* (pMMBpulA:cenB). 12 - 16: *K. oxytoca* (pMMBcenB). 1: Cell-free culture medium. 2, 7, 12: intact cells. 3, 8, 13: cytoplasmic fraction. 4, 9, 14: periplasmic fraction. 5, 10, 15: inner membrane fraction. 6, 11, 16: outer membrane fraction. Cultures grown with maltose: +; without maltose: -. Each lane was loaded with an equal volume of the original culture. Arrowhead indicates full-length PulA:CenB migration. Molecular mass markers (kDa) are indicated on the left.
cells grown with (lane 5) and without (lane 10) maltose supplementation indicated that the chimeric protein associated with the Sec pathway for protein secretion (see INTRODUCTION and Figure 4). This finding is consistent with previously published experiments indicating the requirement of the Sec pathway for PulA secretion (Pugsley et al. 1991). The full-length protein was absent from the periplasmic fraction (lane 4) and present in the outer membrane (lane 6) when cells were grown in the presence of maltose, whereas it was present in the periplasmic fraction (lane 9) and absent from the outer membrane (lane 11) of cells grown without maltose. These results suggest that PulA:CenB interacted with components of the PulA-specific (maltose-induced) secretory pathway, and which were required for translocation to the outer membrane.

A cell-free supernatant sample of a culture grown in the presence of maltose (lane 1) was included and indicated that a very small amount of the 100 kDa protein species was located in the culture medium. This was done to see if PulA:CenB (or proteolytic products) was released from cells. Full-length PulA:CenB and the 100 kDa proteolytic species were also detected in the cell-free medium of cultures grown in TYP without maltose supplementation (see Figure 15, lane 4M). Therefore, the full-length and 100 kDa protein species seen in the culture medium are likely the result of leakage from cells or cell lysis. In Figure 23, the 100 and 47 kDa proteolytic species showed the highest concentration in the cytoplasmic (lanes 3 and 8) and periplasmic fractions (lanes 4 and 9; plus and minus maltose, respectively). If the 100 and 47 kDa species correspond to intact CenB and the C-terminal end of CenB (as described in Section 3.6.4; Figure 22), they would lack a signal peptide and would not be expected to cross the cytoplasmic membrane in a Sec-dependent manner. Therefore, either proteolysis of PulA:CenB occurred in the periplasm as well as the cytoplasm, or the proteolytic products are
translocated from the cytoplasm to the periplasm in a Sec-independent manner.

Quantification of the immunoreactive bands detected in Figure 23 (lane 6) indicated that the intensity of the 200 kDa (PulA:CenB) species in the outer membrane of cells grown in the presence of maltose was ca. 8% the intensity of the 100 or 200 kDa species in unfractionated cells (lane 2). The CMCase data (see Table III) indicated that K. oxytoca (pMMBpulA:cenB) cultures grown in TYP medium with IPTG + maltose supplementation had 20% of the cellulase activity exposed to the extracellular medium. However, the DNS lysis data (Table IV) indicated that 60% of the periplasmic content of these cells were released while approximately 8% of their cytoplasmic content was released. Therefore, it can be concluded that between 32% and 40% of the “intact cell” samples were still intact in the assay. As a result, between 6% and 8% of this 20% CMCase activity can be associated with intact cells. The value of 6-8% medium exposed CMCase activity is similar to the 8% outer membrane localized PulA:CenB protein.

In contrast to the above results obtained with K. oxytoca (pMMBpulA:cenB), when TYP cultures of K. oxytoca (pMMBcenB) induced with 0.1 mM IPTG were subjected to the same fractionation procedure the CenB protein was found to be associated with all fractions (Figure 23 lanes 12 - 16). However, the outer membrane fraction appeared to contain the greatest amount of CenB protein. Since the inner membrane fraction (lane 15) contained CenB, the native CenB signal peptide seems to be recognized by the K. oxytoca Sec pathway machinery, such that the CenB in the inner membrane fraction consisted of proteins in the process of translocation across the inner membrane into the periplasm. This is consistent with a report that CenB can be purified from the periplasm of E. coli cells (Owolabi et al. 1988).
3.7.1 Octyl-POE extraction of outer membrane proteins.

A problem associated with cell fractionation of proteins produced by heterologous or over-expression is that inclusion bodies may co-purify with the outer membrane fraction (Wingfield 1997). In order to assess which of the proteins in outer membrane preparations of *K. oxytoca* (pMMBpulA:cenB) grown in TYP + maltose and *K. oxytoca* (pMMBcenB) TYP-grown cultures were genuine membrane proteins, as opposed to inclusion bodies, outer membrane preparations were extracted with the detergent octyl-POE (0.5%), reported to solubilize proteins from outer membranes but not inclusion bodies (Garavito and Rosenbusch 1986). Proteins that are not solubilized after repeated extractions with a higher concentration of octyl-POE (3%) are thought to be in an insoluble form: i.e., inclusion bodies that co-purified with the outer membrane in the sucrose gradient used in this fractionation procedure. To ensure that there was no carry-over of soluble protein, membrane particles to be treated with octyl-POE were first pelleted in an ultracentrifuge (100 000 X g for 100 minutes). The membrane pellets were resuspended in buffer and the proteins extracted with octyl-POE, separated by SDS-PAGE, and detected by Western immunobloting.

A Western blot of membrane extracts prepared in this way is shown in Figure 24. These results indicate that there was full-length CenB and the 47 kDa proteolytic species associated with the outer membrane of *K. oxytoca* (pMMBcenB) cells (lane 2). However, the predominant species of CenB associated with outer membranes was in inclusion bodies (lane 7). On the basis of extract volume, the proportion of the *K. oxytoca* (pMMBcenB) outer membrane fraction loaded in lane 7 was 0.05 of that loaded in lane 1, and 0.017 of the amount loaded in lanes 2 - 6. Therefore, almost all of the CenB protein isolated from this strain seems to be present as octyl-POE-insoluble inclusion bodies which
Figure 24: Octyl-POE solubilization of *K. oxytoca* (pMMBpulA:cenB) and *K. oxytoca* (pMMBcenB) outer membrane proteins. 1 - 7: *K. oxytoca* (pMMBcenB). 8 - 14: *K. oxytoca* (pMMBpulA:cenB). 1, 8: membrane-free fraction. 2, 9: 0.5% octyl-POE extract. 3, 4, 5, 6, 10, 11, 12, 13: sequential 3% octyl-POE extract. 7, 14: Octyl-POE-insoluble material. Lane 7 was loaded with 0.05 and 0.017 the volumes loaded in lane 1 and lanes 2 - 6, 8 - 14, respectively. Arrowhead indicates full-length PulA:CenB migration. Molecular mass markers (kDa) are indicated on the left.
co-purified with the outer membrane fraction.

Similar to the results obtained with *K. oxytoca* (pMMBcenB), the outer membrane fraction of *K. oxytoca* (pMMBpulA:cenB) also contained anti-CenB immunoreactive protein. In this case the outer membrane fraction contained full-length PulA:CenB and the 100 kDa species (although the latter was in greater abundance; lane 9). Similarly, the *K. oxytoca* (pMMBpulA:cenB) outer membrane fraction subjected to octyl-POE solubilization (lane 14) indicated that a significant proportion of insoluble PulA:CenB protein co-purified with the outer membrane (amount of extract in lane 14 was 0.05 the amount in lane 9).

Therefore, although some PulA:CenB and CenB proteins were solubilized from outer membrane preparations in 0.5% octyl-POE (lanes 2 and 9), a significant portion of PulA:CenB and CenB that co-purified with the outer membranes were present as inclusion bodies based on this method (insoluble in 3% octyl-POE).

Since octyl-POE treatment of the outer membrane fraction indicated that the bulk of the CenB-containing insoluble material copurified with the outer membrane by sucrose density centrifugation, it is possible that the accumulation of CenB in inclusion bodies within cells of *K. oxytoca* (pMMBcenB) disrupted membrane integrity in the CMCase assay, leading to a high “cell-surface” associated CMCase activity due to cell lysis (recorded in Table III and IV).
3.7.2 Analysis of soluble versus insoluble cytoplasmic and periplasmic cellulases.

Since significant portions of PulA:CenB and CenB seemed to form inclusion bodies, it was possible that the cytoplasmic and periplasmic fractions analysed by Western blots were contaminated with insoluble protein. In order to distinguish between soluble and insoluble proteins, the cytoplasmic and periplasmic fractions of *K. oxytoca* (pMMBpulA:cenB) and *K. oxytoca* (pMMBcenB) were subjected to ultracentrifugation at 100 000 X g for 100 minutes to sediment insoluble granules, and the pellets (insoluble particles) and supernatant liquid (soluble proteins) retained separately (Wingfield 1997). A Western blot (Figure 25) developed from SDS-PAGE of these samples indicated that most of the PulA:CenB detected in cytoplasmic (lanes 5 and 6) and periplasmic (lanes 7 and 8) fractions was soluble (lanes 5 and 7; a small amount of cytoplasmic PulA:CenB was insoluble (lane 6).

CenB appeared soluble in the cytoplasm since all of the protein was detected in lane 1 (soluble fraction) and not in lane 2 (ultracentrifugation pellet; Figure 25). A large portion of periplasmic CenB was soluble (lane 3) whereas a smaller but still significant portion was insoluble (lane 4).

The data in this series of experiments indicate that most of the PulA:CenB and CenB proteins (and proteolytic products) detected in cytoplasmic and periplasmic fractions by Western blot are soluble. Therefore, the majority of the insoluble protein fraction had been removed and co-purified with the outer membrane preparation of the strains *K. oxytoca* (pMMBpulA:cenB) and *K. oxytoca* (pMMBcenB) (see Section 3.7.1).
Figure 25: Soluble and insoluble proteins in cytoplasmic and periplasmic fractions of \textit{K. oxytoca} (pMMBpulA:cenB) and \textit{K. oxytoca} (pMMBcenB). 1 - 4: \textit{K. oxytoca} (pMMBcenB). 5 - 8: \textit{K. oxytoca} (pMMBpulA:cenB). 1, 2, 5, and 6: cytoplasmic fractions. 3, 4, 7, and 8: periplasmic fractions. 1, 3, 5, and 7: soluble samples. 2, 4, 6, and 8: insoluble samples. Molecular mass markers (kDa) are indicated on the left side. The arrowhead indicates the migration of full-length PulA:CenB.
3.7.3 Detection of cell surface localized PulA:CenB and CenB using immunofluorescence.

The cell fractionation studies indicated that PulA:CenB and CenB were located in the outer membrane, albeit in low amounts, and so it was of interest to determine if these proteins were exposed to the extracellular medium. Accordingly, indirect immunofluorescence using intact cells and antibodies directed against CenB was used to detect medium-exposed PulA:CenB or CenB. The strains *K. oxytoca* (pMMBpulAxenB), *K. oxytoca* (pMMBcenB), and *K. oxytoca* (pMMB207) were grown, washed, fixed to slides, and probed with anti-CenB antibodies as described in MATERIALS AND METHODS (Section 2.26). The results from one such experiment are summarized in Figure 26. The antibody combination used did not react with *K. oxytoca* proteins as shown by the absence of fluorescence of the control strain *K. oxytoca* (pMMB207) (Panel 2A). Additionally, fluorescence was not detected when intact cells were labelled without the CenB primary antibodies whereas cells that were treated with lysozyme prior to antibody labelling fluoresced as intensely as those shown in Figure 26 (data not shown).

The detection of fluorescence-labelled bacteria of both strains *K. oxytoca* (pMMBpulA:cenB) and *K. oxytoca* (pMMBcenB) might be thought to indicate that the proteins PulA:CenB and CenB are exposed to the extracellular medium. However, the detection of fluorescence regardless of maltose supplementation differs from the results from cell fractionation experiments (see Figure 23) where, in the absence of maltose supplementation (for expression of PulA-specific secretion genes), PulA:CenB was not detected in the outer membrane of *K. oxytoca* (pMMBpulA:cenB) cells. Additionally, CMCase activity
Figure 26: Immunofluorescent detection of surface exposed PulA:CenB and CenB in paraformaldehyde treated cells. Panel 1: bacteria photographed using phase contrast. Panel 2: Fluorescent image of the same bacteria. Images were photographed using 1000 X magnification.
K. oxytoca (pMMB207) IPTG + maltose

K. oxytoca (pMMBcenB) IPTG

K. oxytoca (pMMBcenB) IPTG + maltose

K. oxytoca (pMMBpulA:cenB) IPTG

K. oxytoca (pMMBpulA:cenB) IPTG + maltose
measured using intact cells of *K. oxytoca* (pMMBpulA:cenB) indicated that very little enzymatic activity [15% (IPTG induced) or ca. 6% (IPTG + maltose); see Section 3.5] was exposed to the extracellular medium regardless of maltose supplementation (see Section 4.2.1). This discrepancy suggested that cells might be permeabilized when fixed in paraformaldehyde in preparation for antibody labelling (cells not fixed in paraformaldehyde did not remain anchored to the poly-L-lysine microscope slides used; data not shown).

In order to clarify the discrepancy between the low amounts of surface exposed CMCase activity and detection of surface-localized CenB and PulA:CenB, intact cells in solution (without fixing in paraformaldehyde) were labelled with antibodies as described in Section 2.26 (Figure 27). Low levels of fluorescence were detected in intact cells of *K. oxytoca* (pMMBcenB) +/- maltose supplementation and *K. oxytoca* (pMMBpulA:cenB) supplemented with maltose. Since these photographs were taken using wet mounts some cells appear as halos (different field of focus) and some fluorescing cells appear as streaks (streaming of cells during photographic exposure). The low levels and amounts of fluorescence of samples in Figure 27 indicate that very little PulA:CenB and CenB proteins are accessible to the antibodies used. Therefore, this supports the CMCase results (see Section 3.5) indicating that low amounts of CMCase activity were exposed to the extracellular medium. Furthermore, these data indicate that treatment with paraformaldehyde permeabilizes intact cells such that intracellular CenB and PulA:CenB was accessible to the primary antibodies.

3.8 Analyses of PulA expression and secretion.

After determination of the cellular association and sub-cellular
Figure 27: Immunofluorescent detection of surface exposed PulA:CenB and CenB. Panel 1: bacteria photographed using phase contrast. Panel 2: Fluorescent image of the same bacteria. Images were photographed using 1000 X magnification. Scale bar at upper right corner of phase images represents 10 μm.
*K. oxytoca*(pMMB207) IPTG + maltose

*K. oxytoca*(pMMBcenB) IPTG

*K. oxytoca*(pMMBcenB) IPTG + maltose

*K. oxytoca*(pMMBpulA:cenB) IPTG

*K. oxytoca*(pMMBpulA:cenB) IPTG + maltose
distribution of CMCase activity and CenB immunoreactive species, I measured the distribution of PulA activity in similar experiments to see if *K. oxytoca* (pMMBpulA:cenB) secreted wild type levels of pullulanase. Since PulA-specific antibodies were not available, the presence of PulA was evaluated by use of an enzymatic assay developed using a chromogenic substrate for α-1,6-amylases: Red-pullulan (Megazyme). Pullulanase activities were assayed in cell extracts and intact cells as described in Section 2.26.

The pullulanase specific activities indicate that under the conditions tested, approximately 40% of total cell-associated PulA was present on the cell surface of the control strain *K. oxytoca* (pMMB207) (Table VI). This distribution of PulA activity is similar to previously published reports in which pullulanase activity of *K. oxytoca* was evaluated by measuring the release of reducing sugars from pullulan (d'Enfert et al. 1987).

A decrease in surface exposure of PulA activity in *K. oxytoca* (pMMBpulA:cenB), in which both PulA:CenB and PulA expression were induced, could be indicative of competition between these two proteins for components of the secretory pathway. Alternatively, PulA:CenB may remain associated with the Sec and/or the PulA-specific secretory proteins for a longer time than would PulA. As a result, the rate of PulA secretion would be slowed compared to wild type secretion, and a greater proportion of PulA would remain within the cell. Therefore, the effect of PulA:CenB expression on chromosomally encoded PulA secretion in *K. oxytoca* (pMMBpulA:cenB) cultures was determined using the PulA assay described (Section 2.26). Induction of PulA:CenB expression by maltose resulted in a reduction of surface exposed PulA activity to 12% of the total cell-associated activity (compared to 40% cell-surface exposed
Table VI: Total and cell-surface-located pullulanase activity measured in different strains of *K. oxytoca*.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pullulanase activity¹</th>
<th>0.4% Maltose</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Intact cells</td>
<td>Disrupted cells</td>
</tr>
<tr>
<td><em>K. oxytoca</em> (pMMB207)²</td>
<td></td>
<td>39</td>
<td>61</td>
</tr>
<tr>
<td><em>K. oxytoca</em> (pMMBpulA:cenB)³</td>
<td></td>
<td>12</td>
<td>88</td>
</tr>
</tbody>
</table>

¹Pullulanase activity expressed as a percentage of the total for each sample. Pullulanase activity in uninduced *K. oxytoca* (pMMB207) cultures was 6.1 X 10^-2 (disrupted; 4 X 10^-2) and 6 X 10^-3 (intact cells; 4 X 10^-3) Units/mg total protein.

²Total activity was 4.9 X 10^-1 (<1 X 10^-1) Units/mg total protein.

³Total activity was 2.4 X 10^-1 (<5 X 10^-2) Units/mg total protein.

Numbers within parentheses represent the standard deviation of three assays.
PulA in the control strain *K. oxytoca* (pMMB207); Table VI). This result suggests that expression of the chimeric PulA:CenB interfered with secretion of PulA, possibly by interacting with some component(s) required for PulA secretion. This component could be one or more of the proteins in the Sec pathway and/or the PulA-specific secretory pathway. Furthermore, since the surface exposed PulA activity decreased instead of increased when *pulA:cenB* was expressed simultaneously with *pulA*, these cells did not seem to have lysed in the assay.

3.9 Growth of *K. oxytoca* strains on cellulose.

As stated in the Introduction, the goal of my thesis research was to evaluate the potential of PulA in catalyzing secretion of cellulases as chimeric proteins from *K. oxytoca* cells. Although growth on cellulose these strains was not the immediate goal of my research, it seemed appropriate to determine whether they could grow on cellulose. Growth of *K. oxytoca* cultures on cellulose, by CenB-mediated hydrolysis (either native CenB or PulA:CenB), would confirm that the hydrolytic products of cellulose could be used as carbon sources. Furthermore, growth of *K. oxytoca* (pMMBpulAxenB) on cellulose would indicate whether sufficient PulA:CenB was secreted into the culture medium resulting in growth on cellobiose (a CenB-mediated cellulose hydrolytic product).

3.9.1 Growth of *K. oxytoca* cultures by CenB-mediated hydrolysis of cellulose.

An evaluation of *K. oxytoca* growth based on CenB-dependent cellulose degradation was done in a minimal liquid medium containing CMC or avicel in the presence of 0.1 mg/ml of aseptic purified
CenB (filtered through a 0.2 μm pore size filter).

Growth of *K. oxytoca* in minimal medium with various amounts of cellobiose as the sole carbon source (0% to 0.4%) indicated that 0.075% cellobiose, present in the medium at the time of inoculation, would be sufficient for a culture to grow from 8 Klett units (3 X 10^7 cells/ml) to 100 Klett units (3.7 X 10^8 cells/ml) within 24 hours. Previous experiments with CenB had indicated that 0.1 mg CenB/ml included in a suspension containing 10 mg avicel would produce a solution containing 0.075% cellobiose within 18 hours (A. Meinke, personal communication). Although the specific activity of CenB on the soluble cellulose derivative CMC (928 μmole glucose released/μmol CenB/min) is much greater than its activity on the insoluble cellulose avicel (2.22 μmole glucose released/μmol CenB/min; Tomme *et al.* 1996), CenB hydrolysis of CMC would result in the production of carboxymethylated sugars including carboxymethyl-cellobiose (CMC is derivatized from cellulose by carboxy-methylation on 65% - 90% of the glucose subunits; Sigma Technical Service). As I was unsure of the effects of carboxymethyl-cellobiose on growth of *K. oxytoca*, growth on both CMC and avicel was tested.

The growth of *K. oxytoca* in minimal M63 medium supplemented with either 0.4% CMC or avicel as the sole source of carbon and additionally supplemented with 0.1 mg/ml of purified CenB was monitored (Figure 28). It was found that hydrolysis of avicel by CenB provided enough solubilized substrate to allow almost one culture doubling (26 to 43 Klett units) in 24 hours, whereas hydrolysis of CMC resulted in a lower level of growth in the same time period (26 to 34 Klett units). Therefore, hydrolysis of CMC or avicel by this amount of CenB produced enough cellobiose to support a small amount of growth of *K. oxytoca*. The decline in cell concentration in avicel medium is likely
Figure 28: Growth curve of *K. oxytoca* in M63 basal salts and cellulose as the carbon source. 0.1 mg/ml purified CenB was added to some cultures at the time of inoculation.
the result of adsorption of cells to the insoluble avicel substrate which settled out from the solution. (30 minutes was allowed to elapse between vortexing of avicel cultures and measurement of turbidity to reduce light-scatter by suspended avicel solids.) If it is assumed that *K. oxytoca* cells bound similarly to avicel when CenB was present, perhaps even more growth would have been observed than was indicated by culture turbidity.

However, unlike a situation in which an increasing culture density of cells that continuously produced CenB, which would result in an increasing concentration of CenB over time, in this experiment the amount of CenB and thus the rate of CenB-catalysed hydrolysis of cellulose was fixed, and would limit the extent of growth of the culture. Therefore, in this experiment the amount of cellobiose produced would limit growth, and growth kinetics were not expected to be analogous to those obtained when M63 medium containing 0.075% cellobiose as the carbon source was inoculated with *K. oxytoca* cells.

3.9.2 Attempts to isolate spontaneous mutants of *K. oxytoca* (pMMBpulA:cenB) or *K. oxytoca* (pMMBcenB) that could grow on cellulose.

Although *K. oxytoca* cultures grew on cellulose when supplemented with CenB, neither *K. oxytoca* (pMMBpulA:cenB) nor *K. oxytoca* (pMMBcenB) grew on cellulose as the sole carbon source (data not shown). Therefore, attempts to isolate spontaneous mutants of *K. oxytoca* (pMMBpulA:cenB) cultures able to grow on cellulose were made. It was thought that if a mutant could be isolated, identification of the mutant gene might provide information about the block in secretion.

Cultures of *K. oxytoca* (pMMBpulA:cenB) and *K. oxytoca*
(pMMBcenB) were grown in M63 medium with maltose as the carbon source (maltose also ensured induction of PulA:CenB and all the PulA-specific secretory proteins) and used to inoculate liquid media containing CMC or avicel as the carbon source (+/- 0.1% maltose supplementation for the induction of the PulA-specific secretory proteins) to an initial culture density of 10^7 cells/ml. The concentration of maltose used (determined from a titration experiment using 0% to 0.4% maltose as sole carbon source in minimal medium; data not shown) was sufficient to allow approximately two culture doublings (from ca. 5 X 10^7 to 3 X 10^8 cells/ml). Growth was followed through three successive transfers in which each culture was monitored for about 45 days. An experiment run in parallel included 0.1 mM IPTG for induction of pulA:cenB or cenB. Although these cultures (+/- IPTG) grew to the maximum density supported by maltose (3 X 10^8 cells/ml), they did not grow to a higher density (data not shown). Since this enrichment strategy for spontaneous mutants able to grow on cellulose was not successful, it appeared that either multiple mutations were required or the single site spontaneous mutation frequency was less than 1 in 8 X 10^7 (total number of cells in the primary inoculum).

Other experiments were done to select spontaneous mutants on a solid medium. In these experiments, the K. oxytoca (pMMBpulA:cenB) and K. oxytoca (pMMBcenB) inocula were grown as described above for selection in liquid media. The cell density of the inoculum cultures were measured and appropriate dilutions made so that 10^6, 10^7, and 10^8 cells were mixed with molten M63 agarose (45 - 50° C) containing 0.1 mM IPTG, and 0.4% avicel, CMC, or cellobiose (used as a control for cell viability) as carbon sources and overlayed on an agarose base containing the corresponding carbon source and appropriate supplements. To reduce background growth, maltose (inducer and carbon source) was omitted from the selection medium. After a 4 day
incubation period at 30° C, growth on the cellobiose containing medium produced a lawn of cells as expected, and colonies appeared on the CMC and the avicel selection plates: 14 and 5 colonies, respectively (no growth on cellulose was observed for K. oxytoca (pMMBcenB) cultures). Three colonies from both the CMC and avicel plate were resuspended in 1 ml M63 (no carbon source) and spread onto fresh plates containing the appropriate carbon source and supplements. The colonies that formed from these 6 isolates (after a two day incubation period; 9 colonies from the CMC plates and 5 from avicel plates) were inoculated into corresponding liquid media (i.e., from a CMC agarose plate into CMC liquid medium, etc.); elimination of agarose and any potential impurities it might contain would be a more rigorous test for growth on cellulose. None of the colonies that arose on plates grew in the corresponding liquid medium (after 7 days), although they did grow in M63 medium containing glycerol. Therefore, I conclude that the colonies that arose on these plates were spontaneous mutants growing on a component present in the agarose used to prepare the plates.

The selective plates described above were kept at 30° C for about 4 more weeks, during which time they were scrutinized for the appearance of additional colonies or the increase in size of existing colonies, indicative of the occurrence of a mutation allowing growth on cellulose. During this period there were no changes in the colonies on the cellulose selection plates that would indicate the occurrence of a mutation allowing growth on cellulose.

3.9.3 Chemically induced mutagenesis of K. oxytoca (pMMBpulA:cenB) and selection for growth on cellulose.

The experiments described above were done to see if the spontaneous mutation frequency was high enough to allow the isolation of
a strain of *K. oxytoca* (pMMBpulA:cenB) able to grow on cellulose as the sole source of carbon. Since negative results were obtained, a mutagen was used to increase the mutation frequency. Therefore, MNNG induced mutagenesis was used to generate mutants of *K. oxytoca* (pMMBpulA:cenB) from which those that could grow on cellulose would be selected. *K. oxytoca* (pMMBpulA:cenB) cells (10⁷) were exposed to 4 different concentrations of MNNG (13.2, 50, 100, and 1000 µg/ml) for 0, 15, 30, and 45 minutes as described (Section 2.24). Mutagenesis survival and mutation frequency to rifampicin resistance (Rif⁺) were monitored for each time point (one representative experiment is shown in Figure 29).

The mutagenesis survival of *K. oxytoca* (40% survival at 30 minutes; Figure 29A) was better than survival of *E. coli* cells similarly treated (50 µg/ml MNNG; 10% survival at 30 minutes; *i.e.*, fewer *K. oxytoca* cells were killed by the mutagenesis regime; Miller 1992). Additionally, the frequency of mutation to Rif⁺ in *E. coli* (10⁻³ after 5 minutes mutagenesis) was higher than the Rif⁺ frequency measured for *K. oxytoca* (10⁻⁵ after 5 minutes of mutagenesis; Figure 29B). The Rif⁺ mutation frequency and percent survival measured in this experiment were not affected by increasing the concentration of MNNG (up to 1000 µg/ml). A fresh sample of MNNG was purchased and found to produce the same results. Therefore, it appeared that *K. oxytoca* cells were more resistant to MNNG mutagenesis than *E. coli* cells.

However, since Rif⁺ mutations did occur at a frequency above the spontaneous frequency (Figure 29B), I attempted a selection for mutations allowing growth on cellulose. After a 24 hour outgrowth period on TYP medium, 10⁸ cells from each mutagenesis time point were spread onto plates or inoculated into liquid medium containing avicel or CMC as carbon source [M63 medium containing 0.1 mM IPTG and 0.1%
Figure 29: Example of cell killing and the frequency of mutation in *K. oxytoca* (pMMBpulA:cenB) exposed to 50 μg/ml MNNG. A: MNNG mutagenesis survival curve. B: Frequency of mutation to rifampicin resistance.
maltose (for the induction of the PulA-specific secretory proteins)] and incubated until there were visible colonies on the solid medium or visible turbidity in the liquid medium. Liquid cultures inoculated from all mutagenesis time points grew from ca. 10 Klett units (4 X 10^7 cells/ml) to ca. 100 Klett units (3.7 X 10^8 cells/ml). This extent of growth would be expected for *K. oxytoca* growing on 0.1% maltose alone (See section 3.9.2). Therefore, there was no detectable growth on cellulose of mutated *K. oxytoca* (pMMBpulA:cenB) in liquid media at all time points tested. A total of 18 colonies were isolated on CMC selection plates (6 from the 5 minute time point, 4 from the 15 minute time point, 3 from the 30 minute time point, and 5 from the 45 minute time point). Cells from these colonies were grown overnight in liquid M63-glycerol medium and 5 µL (ca. 10^6 cells) of these cultures deposited onto agarose plates with CMC or avicel as the carbon source, and incubated overnight. Of the original 18 isolates, 9 grew on these plates (either on CMC or avicel). These 9 were streaked onto fresh agarose plates containing CMC or avicel. I found that 7 isolates grew (on avicel or CMC). However, when transferred to liquid media of the same composition, none of the 7 isolates grew, although growth was observed on M63-cellobiose control media. Therefore, I conclude that these isolates were probably growing on a component of the agarose used to prepare these plates. These results are similar to those observed for the selection of potential spontaneous mutants on CMC or avicel plates discussed in Section 3.9.2 (similar results were obtained when the 24 hour outgrowth period was omitted from the MNNG mutagenesis procedure).

In summary, MNNG mutagenesis of *K. oxytoca* (pMMBpulA:cenB) increased the Rif^r mutation frequency from 1 in 10^8 to 1 in 10^5, but this mutation frequency was not high enough to isolate strains allowing growth on cellulose, suggesting a requirement for
multiple mutations.

3.9.4 Unusual growth kinetics of *K. oxytoca* (pMMBpulA:cenB) in M63 medium.

I discovered in growth studies that cultures of *K. oxytoca* (pMMBpulA:cenB) grown in M63 (0.4% glycerol) medium in which PulA:CenB expression was induced by the addition of either IPTG or maltose exhibited a triphasic growth cycle (Figure 30). This triphasic growth pattern is different from the growth of this strain in TYP medium where a classical exponential phase was followed by a high cell density (ca. 10^9 cells/ml) stationary phase. Induced cultures in M63 medium initially grew with wild type kinetics, but the culture turbidity then decreased between 4 and 10 hours after induction, after which the cultures appeared to recover and resume growing to the maximum wild type density (ca. 300 Klett units, ca. 10^9 cells/ml) after 24 hours (cells from such a culture are hereafter referred to as “survivors”. The reason for decline in culture turbidity was not clear; although it was likely to be cell lysis, plasmid DNA was not detected in the culture medium; data not shown). The decline in culture turbidity was more acute with maltose than with IPTG induction. This phenotype was not observed when *K. oxytoca* (pMMB207) cultures were grown in M63 medium under the same conditions, suggesting that this phenotype is not simply due to components in the culture medium, but instead indicates that accumulation of PulA:CenB in this minimal medium inhibits growth. *K. oxytoca* (pMMBcenB) grown in M63 with IPTG (maltose is not an inducer of cenB expression) exhibited a growth pattern similar to that seen for *K. oxytoca* (pMMBpulA:cenB) grown on M63 with IPTG but with a less acute decline than was observed in M63 with maltose. Therefore, it appeared that a high level of CenB (IPTG induction) and PulA:CenB (maltose or IPTG induction) expression resulted in a multi-phasic growth
Figure 30: Growth kinetics of *K. oxytoca* (pMMBpulA:cenB) (A) and *K. oxytoca* (pMMBcenB) (B) in a glycerol minimal M63 medium. The inducer IPTG (0.1 mM) was added at mid-logarithmic phase (40 - 50 Klett units) whereas maltose (0.4%) was added with the inoculum.
pattern that was not observed when the cultures were grown in TYP medium.

3.9.5 Western blot analysis of PulA:CenB accumulation in *K. oxytoca* (pMMBpulA:cenB) grown in glycerol minimal M63 medium and induced with IPTG or maltose.

The accumulation of PulA:CenB during the triphasic growth cycle of *K. oxytoca* (pMMBpulA:cenB) grown on M63 and induced with IPTG or maltose was investigated by Western blot analysis following SDS-PAGE. *K. oxytoca* (pMMBpulA:cenB) was grown in M63 and TYP medium, and PulA:CenB expression induced with either IPTG (added at mid-logarithmic phase) or maltose (added with the inoculum). Culture samples were taken at various time points before and after the drop in cell density (which occurred about 4 hours after culture induction), proteins separated by SDS-PAGE, and identified by Western blot using anti-CenB antibodies (Figure 31). Maltose induced cultures were sampled at 3, 4, 4.5, 5, 6, and 7 hours after inoculation. IPTG-induced cultures were sampled at 0, 3, 4, and 5 hours after induction.

The Western blots shown in Figure 31 indicate that all cultures accumulated full-length PulA:CenB as well as the 100 and 47 kDa proteolytic products, indicating that the fusion gene was not deleted or expression eliminated by a promoter mutation. Additionally, M63 grown cultures during the decline phase (5, 6, and 7 hour IPTG or 3, 4, and 5 hour maltose samples; panel A) did not accumulate significantly less CenB immunoreactive proteins nor did they secrete more PulA:CenB than the same strains grown in TYP medium and samples analysed at the same time points (panel B). However, two differences between TYP and M63 grown cultures are apparent. In the first instance the kinetics of
Figure 31: Accumulation of PulA:CenB in triphasic cultures of *K. oxytoca* (pMMBpulA:cenB). A: M63 medium. B: TYP medium. Cultures were induced with 0.1 mM IPTG added at mid-logarithmic phase (35 - 55 Klett units) or with 0.4% maltose added with the inoculum. C: intact cells. M: cell-free culture medium. The numbers above the lanes correspond to the sampling time (in hours after addition of inducer). Molecular mass markers (kDa) are indicated on the left. The arrowhead indicates the migration of full-length PulA:CenB. The equivalent of 1 X 10^7 cells were loaded in each lane.
proteolysis appears different. M63 grown cultures appeared to accumulate full-length PulA:CenB and the 100 kDa proteolytic product in equal amounts. In contrast, in TYP grown cultures proteolysis of full-length protein into the 100 kDa species was very rapid, such that the latter accumulated before there was detectable full-length PulA:CenB. Additionally, in M63 medium two bands of equal intensity were detected above the 83 kDa marker, i.e., the 100 kDa proteolytic product. However, in TYP, if there were two species (as in the TYP + IPTG samples), the higher molecular mass one appeared in greater abundance than the lower one. Secondly, when M63 cultures were induced with maltose, full-length PulA:CenB accumulated at an earlier time point (3 hours) than in TYP grown cultures (5 hours). Therefore, in induced M63 cultures, full-length PulA:CenB accumulated earlier and some of the full-length protein was degraded into two equally abundant but slightly differently sized species of 100 kDa.

The appearance of the K. oxytoca (pMMBpulA:cenB) "survivors" indicated that a secondary mutation in the secretory pathway or a mutation preventing the accumulation of inclusion bodies may have occurred, potentially resulting in increased secretion of PulA:CenB. However, it was also possible that these cultures had either lost the plasmid or expression of the fusion gene had been reduced or eliminated by a promoter mutation or loss of the pulA:cenB fusion gene. As "survivor" strains produced Congo red clearings (data not shown), and PulA:CenB was detected in cell lysates by Western blot analysis (see Section 3.9.5), and the entire pMMBpulA:cenB plasmid was present (data not shown), it was not likely that the fusion gene was deleted or that a down-promoter mutation occurred. Therefore, these results suggested that "survivors" had overcome an as yet unknown detrimental effect of PulA:CenB expression and/or secretion.
As a result of the above interpretation of the appearance of "survivors", I thought mutants able to grow on cellulose as a carbon source might be enriched from amongst them. "Survivors" ($10^7$ cells) were inoculated into M63 medium supplemented with cellulose as described above. It was thought that if there were cells able to grow on cellulose this approach would enrich for them until they dominated the culture population. This approach, however did not result in the enrichment of cells able to grow on cellulose, similar to the results obtained for the enrichment of spontaneous mutants able to grow on cellulose described above (Section 3.9.2). Therefore, whatever factors result in the appearance of "survivors" do not result in enhanced levels of enzymatically active PulA:CenB secretion.

3.10 Evaluation of four different S-linked analogues of maltose.

The maltose regulon system has been extensively studied in *E. coli* and it was found that maltose can be polymerized into longer maltodextrins by the action of MalQ (an amylomaltase), and maltodextrins can have their chain lengths reduced to that of maltose by MalZ (a maltodextrin glucosidase; Decker *et al.* 1993). It was reported that *E. coli* can synthesize maltose and maltotriose endogenously from glucose by a complex network of reactions involving at least the two enzymes MalZ and MalQ. These two enzymes are thought to work in concert to maintain low levels of intracellular maltose and maltotriose during growth on other carbon sources, presumably to ensure that the cell is poised to degrade maltose if it should become available. Therefore, exogenously supplied maltose can be polymerized intracellularly by MalQ to maltotriose and longer chain maltodextrins by the addition of glucose to the nonreducing ends. Maltodextrins thus formed could in turn be depolymerized to maltotriose by MalZ (Decker *et al.* 1993). Thus in this scheme an external source of maltose would
increase the internal concentration of maltotriose.

My experiments on maltose induction of pulA and the PulA-specific secretion apparatus, as well as for selection for growth on cellulose were complicated by the fact that maltose is also a carbon source for growth. Since maltose was essential for PulA:CenB secretion, it was difficult to distinguish between growth on maltose and growth on cellulose when both were present in the growth medium. Theoretically, the use of a non-metabolizable maltose analogue equivalent to the lactose analogue IPTG would overcome this problem (Herzenberg 1959). In addition to being non-metabolizable, an appropriate maltose analogue would still bind to the positive regulatory protein MalT, and the MalT/analogue complex should stimulate transcription of maltose regulon genes as does the MalT/maltose complex in the wild type situation (Michaelis et al. 1985). Therefore, four thio analogues of maltose were synthesized (by L. Mackenzie, a student of Dr. S. Withers in the UBC Dept. of Chemistry; Figure 32) and evaluated by me for their ability to induce expression of a MalT regulated promoter (PpulA is one example of a MalT regulated promoter; d'Enfert et al. 1987).

In order to prevent polymerization of the thio analogues into longer maltodextrins as well as stabilize the anomic carbon in the α configuration, two analogues were synthesized with methyl substitutions at the C1 anomeric carbon (analogues 1 and 2; Figure 32). Two analogues were synthesized with α-1,6 linkages (analogues 2 and 3; Figure 32) in order to mimic the branched portion of pullulan [α-1,6-linked maltotriose (α-1,4-linked glucose trisaccharide) subunits]. The analogue most closely resembling maltose was analogue 4 (Figure 32), in which the oxygen atom in the α-1,4 linkage between glucose residues of maltose was replaced with a sulfur atom.
Figure 32: Chemical structures of maltose and 4 thio analogues of maltose. Analogue 1: Methyl-4-S-(\(\alpha\)-D-glucopyranosyl)-4-thio-\(\alpha\)-D-glucopyranoside. Analogue 2: Methyl-6-S-(\(\alpha\)-D-glucopyranosyl)-6-thio-\(\alpha\)-D-glucopyranoside. Analogue 3: 6-S-(\(\alpha\)-D-glucopyranosyl)-6-thio-\(\alpha\)-D-glucopyranoside. Analogue 4: 4-S-(\(\alpha\)-D-glucopyranosyl)-4-thio-\(\alpha\)-D-glucopyranoside. Me: Methyl substituent. Squiggle indicates a mixture of both \(\alpha\) and \(\beta\) configurations. Thio analogues 2 and 3 have an \(\alpha\)-1,6 linkage. Note, for simplicity, adjacent glucose molecules are not shown rotated 180\(^\circ\) from each other.
To evaluate the potential of using each of the four thio-maltose analogues as inducers of maltose regulated promoters, the *E. coli* strain pop3125 (which contains a chromosomal fusion between *malP'* and 'lacZY') was studied (Débarbouillé *et al.* 1978). Since transcription of *lacZ* in *E. coli* pop3125 is dependent on *malP* transcription, which at the time these experiments were done was thought by me to be enhanced by binding of MalT/maltose to P*malP*, induction of expression by maltose and thio analogues (through MalT) was monitored by measuring β-galactosidase activity. *E. coli* pop3125 grown in M63 (glycerol as a carbon source) was used to inoculate media of the same composition, supplemented with 0, 0.004%, 0.01%, 0.04%, 0.1% and 0.4% of each thio analogue of maltose, and incubated overnight (ca. 16 hours) at 30° C. Cells were pelleted, washed, resuspended in Z buffer, and β-galactosidase activity assayed as described (Section 2.17). The averages of the results of two independent experiments are summarized in Table VII. These results indicate that thio-maltose 4 (4-S-(α-D-glucopyranosyl)-4-thio-α-D-glucopyranoside) was the only maltose analogue that greatly increased β-galactosidase activity: an almost 3-fold difference between the uninduced condition (32.7 - 36.5 Miller units) and induction with 0.4% analogue 4 (94.7 Miller units) was found. None of the other three thio-maltose analogues resulted in β-galactosidase activity levels that were greatly higher than the uninduced condition. However, maltose was a better inducer than compound 4, since it yielded 7.7 - fold induction (252.0 Miller units with 0.4% maltose) of β-galactosidase activity compared to the uninduced state.

Although maltose is typically used to induce transcription initiated at promoters of the maltose regulon (including induction of P*pullA* and P*pulC-O*; maltose was used exclusively as the *in vivo* inducer in the experiments described in this thesis), as previously
Table VII: β-galactosidase activity measured in *E. coli* pop3125 in response to various inducers.

<table>
<thead>
<tr>
<th>Inducer (%)</th>
<th>Maltose</th>
<th>Maltotriose</th>
<th>Analogue 1</th>
<th>Analogue 2</th>
<th>Analogue 3</th>
<th>Analogue 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>32.7 (5.8)</td>
<td>36.5 (0.7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.001</td>
<td>31.0 (3.0)</td>
<td>35.4 (2.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.002</td>
<td>35.7 (3.2)</td>
<td>40.4 (2.2)</td>
<td></td>
<td></td>
<td></td>
<td>47.2 (1.9)</td>
</tr>
<tr>
<td>0.004</td>
<td>43.7 (3.4)</td>
<td>43.9 (3.1)</td>
<td>25.7 (13.2)</td>
<td>29.8 (4.1)</td>
<td>30.7 (3.2)</td>
<td>56.7 (1.3)</td>
</tr>
<tr>
<td>0.01</td>
<td>93.4 (6.9)</td>
<td>69.7 (6.3)</td>
<td>26.8 (1.7)</td>
<td>33.1 (1.6)</td>
<td>31.7 (4.6)</td>
<td>81.0 (7.6)</td>
</tr>
<tr>
<td>0.04</td>
<td>161.6 (1.7)</td>
<td>153.8 (8.2)</td>
<td>28.1 (3.3)</td>
<td>30.5 (11.2)</td>
<td>31.9 (5.8)</td>
<td>79.4 (6.2)</td>
</tr>
<tr>
<td>0.1</td>
<td>161.3 (27.0)</td>
<td>209.9 (32.7)</td>
<td>30.0 (1.6)</td>
<td>31.4 (0.26)</td>
<td>28.8 (1.3)</td>
<td>76.1 (4.1)</td>
</tr>
<tr>
<td>0.4</td>
<td>252.0 (20.6)</td>
<td>271.6 (10.2)</td>
<td>36.9 (3.3)</td>
<td>23.0 (0.92)</td>
<td>22.0 (2.1)</td>
<td>94.7 (8.5)</td>
</tr>
</tbody>
</table>

1β-galactosidase activity expressed as Miller units; numbers within parentheses are standard deviations of 4 assays.

Analogue 1: Methyl-4-S-(α-D-glucopyranosyl)-4-thio-α-D-glucopyranoside.
Analogue 2: Methyl-6-S-(α-D-glucopyranosyl)-6-thio-α-D-glucopyranoside.
Analogue 3: 6-S-(α-D-glucopyranosyl)-6-thio-α-D-glucopyranoside.
Analogue 4: 4-S-(α-D-glucopyranosyl)-4-thio-α-D-glucopyranoside.
mentioned the true inducer of this system has been shown to be maltotriose (maltotriose is the species that binds to MalT; Raibaud and Richet 1987). Unfortunately, I was unaware of this publication at the time that the thio analogues of maltose were designed, which is why analogues 1 to 4 were tested. Evaluation of maltotriose as an inducer indicated that maltotriose was as effective an inducer of β-galactosidase activity in *E. coli* strain pop3125 (uninduced 36.5 Miller units versus 271.6 Miller units with 0.4% maltotriose; Table VII) as was maltose, and both were better inducers of β-galactosidase activity than was analogue 4. As previously mentioned, *E. coli* is thought to be able to convert maltose into maltotriose by an interplay between the enzymes MalQ and MalZ (Decker *et al.* 1993). Therefore, it was likely that maltose was as effective an inducer of expression of the PulA-specific secretory genes as was maltotriose.

The *lacZ* insertion in *malP* in strain pop3125 is expected by polarity to disrupt *malQ* expression as well (Pugsley and Dubreuil 1988). Since my data indicate that β-galactosidase activity is still inducible by maltose and analogue 4, these sugars must be converted into the triose form. A novel enzyme, tentatively called maltose-maltotriose phosphorylase (MP), has been proposed to exist and to polymerize glucose residues onto glucodextrins (> 1 glucose residue long; Decker *et al.* 1993). This proposed enzyme (MP) has not been isolated, nor has its existence been shown directly. Since analogue 4 was an effective inducer whereas analogue 1 was not, it is intriguing to suggest that MP exists, and functions to add one glucose molecule onto the non-reducing end of glucodextrins.

In view of these data and the exciting possibility of MP being a key component in regulation of the maltose regulon, we have established
a collaboration with Dr. W. Boos (University of Konstanz, Germany). Initial experiments will determine the kinetics of uptake of the 4 thio maltose analogues tested. Following this, experiments will be designed to determine if analogue 4 is itself an inducer or whether it is converted into an inducer by MP.
4.0 DISCUSSION

The effectiveness of the amino-terminal portion of PulA as a determinant for the secretion of *C. fimi* cellulases from *K. oxytoca* cells is discussed below. Additionally, the choice of CenB as the cellulase in fusions to PulA is also discussed.


At the start of this research, fusion of *cenA* and *cex* to the *K. oxytoca* *pulA* gene appeared to be a promising strategy to obtain secretion of these cellulases. *E. coli* and *K. oxytoca* strains expressing either intact genes or PulA chimeras of CenA and Cex were created, and the Congo red plate assay of CenA (Figure 10) and the MUC plate assay of Cex (Figure 11) indicated that both fusion gene constructions resulted in the production of enzymatically active chimeric proteins.

However, prior to a complete evaluation of PulA:CenA and PulA:Cex secretion from *K. oxytoca*, both *pulA:cenA* and *pulA:cex* should be subcloned into a vector such as pMMB207, which is stably maintained in this host cell. This is because both CenA and Cex are thought to function synergistically to degrade cellulose to cellobiose (Gilkes *et al.* 1991), and so both *cenA* and *cex* would need to be expressed in *K. oxytoca* cells and the resulting proteins secreted from the same culture, ideally in the same cell to eventually obtain growth on cellulose. Expression of both chimeric genes in the same host cell could be most effectively accomplished by subcloning both fusion genes onto the same plasmid. The pre-existing divergent promoter upstream of *pulA* on pUC13pulA:cenA (see Figure 6) could be exploited for this
construction, using existing or newly created appropriate restriction sites. In this way, *pulA:cex* could be subcloned upstream and in the opposite orientation, of *pulA:cenA*. If this avenue (simultaneous expression of *cenA* and *cex* fusion genes for cellulose degradation) were to be investigated further, it would be necessary to determine the conditions for optimal expression of fusion *pulA:cenA* and *pulA:cex* genes and secretion of fusion proteins. Additionally, it would be useful to purify the fusion proteins for detailed enzymological studies. For example, comparisons of specific activities with the native enzymes and, perhaps, additional mutagenesis to enhance catalytic activity and/or secretion. It would be interesting to explore these avenues, for comparison with my work on CenB constructs.

4.2 Choice of CenB for detailed analysis.

Experiments on CenB showed that this enzyme degraded cellohexaose to cellobiose and cellotetraose, and in turn cellotetraose was hydrolysed to cellobiose (by an as yet uncharacterised mechanism; Meinke et al. 1991). Additionally, it was demonstrated that CenB depolymerised 40% of a cotton substrate within 48 hours of incubation (4 nmol CenB/mg cotton), or 48% of the initial amount of bacterial cellulose in a reaction mixture within 48 hours (1 nmol CenB/mg cellulose; bacterial cellulose purified from *Acetobacter xylinum*; Kleman-Leyer et al. 1994). Furthermore, when CenB was mixed with *A. xylinum* bacterial microcrystalline cellulose (BMCC) 73% of the cellulose was solubilized within 24 hours (1 nmol CenB/mg BMCC; Kleman-Leyer et al. 1994). Thus it appears that CenB is able to hydrolyse cellulose from cotton or BMCC to soluble oligosaccharides, ultimately yielding cellobiose. Since CenB can degrade a variety of celluloses to cellobiose, and it would be easier to study one gene (encoding a single protein) as opposed to two genes (the *cenA* and *cex* genes), and the enzymes they
encode, it would seem that CenB presented the greatest applied potential for the long term goal of degradation of cellulose to allow growth of *K. oxytoca* on cellobiose.

4.2.1 Analysis of *pulA:cenB* expression in *K. oxytoca*.

The *pulA:cenB* chimeric gene in pMMBpulA:cenB was subcloned downstream of two inducible promoters (PpulA or Ptac; Figure 8), and therefore the addition of either maltose or IPTG should be sufficient for high-level expression of *pulA:cenB*. Moreover, maltose is required for expression in *K. oxytoca* of the chromosomally encoded genes (the *pulC-O* operon and *pulS*) required for secretion of PulA and simultaneously PulA:CenB.

*K. oxytoca* (pMMBpulA:cenB) cells produced clearings on Congo red stained CMC plates, indicative of release, by cell lysis and/or inefficient secretion, of PulA:CenB from cells and enzymatic hydrolysis of cellulose (Figure 13). Zymogram analysis (Figure 18) and CMCase activities of full-length PulA:CenB and the 100 kDa proteolytic species (Figure 20) provided indirect evidence suggesting that the full-length PulA:CenB protein had about the same CMCase specific activity (within a broad range of experimental error) as the 100 kDa proteolytic species. Therefore, the CMCase activity measured in cultures of *K. oxytoca* (pMMBpulA:cenB) (Table III) was likely the sum of the individual activities of both the full-length PulA:CenB and the 100 kDa species.

Western blot data (Figure 15) indicated that *K. oxytoca* (pMMBpulA:cenB) cells produced a protein that is the size predicted for full-length PulA:CenB (200 kDa). Maximum accumulation of PulA:CenB (17 mg/L) occurred following a 5 hour induction with 0.1 mM IPTG (most of this was retained within cells). Although cellulase activity was
associated with the strains *K. oxytoca* (pMMBpulA:cenB) and *K. oxytoca* (pMMBcenB) (Table III), most of the surface exposed activity (measured on intact cells) was a result of cell membrane disruption as intact cells also displayed a high level of β-galactosidase and PhoA activity (Table IV) as a result of incubation with the CMCase assay substrate. Indeed, 34% (IPTG-induced) or 74% (IPTG + maltose-induced) of the intracellular β-galactosidase activity was measured in supposedly intact cells of *K. oxytoca* (pMMBcenB) cultures. Additionally, intact cells of *K. oxytoca* (pMMBcenB) released significant levels of PhoA activity during the CMCase assay, 100% (IPTG + maltose-induced) or 73% (IPTG-induced). Therefore, a conservative analysis of the CMCase, β-galactosidase, and PhoA activity data indicates that no *K. oxytoca* (pMMBcenB) cells remained intact during the CMCase assay suggesting that all the CMcase activity measured on *K. oxytoca* (pMMBcenB) intact cells was due to release of intracellular CenB.

On the other hand, *K. oxytoca* (pMMBpulA:cenB) intact cell samples appeared to remain intact as less than 10% of intracellular β-galactosidase activity was released (see Table IV). Nevertheless, a significant proportion of *K. oxytoca* (pMMBpulA:cenB) intact cells released PhoA activity (ca. 17% IPTG-induced and ca. 60% IPTG + maltose-induced). A conservative estimate of *K. oxytoca* (pMMBpulA:cenB) in the CMCase assay indicates that 32 - 40% (IPTG + maltose-induced) or 81-83% (IPTG-induced) remained intact. Therefore, 6% (IPTG + maltose-induced) and 16% (IPTG-induced) of the total cell-associated CMCase activity measured (Table III) can be attributed to cells that remained intact during the assay.

*K. oxytoca* (pMMB207) intact cell samples were not disrupted in the CMCase assay (β-galactosidase and PhoA activities
released were less than 10% of intracellular activity).

The release of intracellular β-galactosidase and PhoA activity during the CMCase assay indicates that very little CMCase activity (associated with pulA:cenB or cenB expression) is localized to the outer surface of K. oxytoca cells. Furthermore, the low amount of cell-surface localized CMCase activity (ca. 6%) associated with pulA:cenB expression is proportional to the levels of PulA:CenB detected in outer membrane fractions (ca. 8%) of K. oxytoca (pMMBpulA:cenB) cultures (see Section 4.3 and Figure 23).

4.2.2 Analysis of PulA:CenB proteolytic products.

Along with the presence of full-length PulA:CenB, two degradation products (the 100 and 47 kDa fragments) were prominent and thus of particular interest. Both the 100 and 47 kDa species were detected in samples prepared for electrophoresis by boiling of intact cells in SDS-PAGE loading buffer immediately after harvest and subsequent evaluation in Western blots using anti-CenB antibodies (Figure 15), and both protein species bound to avicel (Figure 16). Taken together, these data suggest that proteolysis of PulA:CenB occurs in vivo at two preferred sites, between the two halves of the chimera (releasing the 100 kDa species CenB; see Figure 22) and between the amino-terminal-proximal fibronectin-like region and the Bacillus-type CBD (releasing the C-terminal 47 kDa CBD; see Figure 22). N-terminal sequencing of the 100 kDa species yielded ambiguous sequence data, suggesting that the sample was a mixture of at least two proteins. Therefore, proteolysis probably does not occur at discrete sequences but within regions predicted to be flexible or exposed (Meinke et al. 1992).
Confirmation of the amino acid sequences of the 100 kDa and 47 kDa proteins should be determined in order to evaluate the possibility of modifying the proteins, making them more resistant to proteolysis. Careful separation of the different protein species could be accomplished in one of the following ways. Empirical, systematic modification of the 2D-electrophoretic conditions used might result in sufficiently abundant and resolved protein species to allow N-terminal sequencing. Alternative protein purification methods, such as gel-filtration or ion-exchange chromatography, perhaps in parallel with 2-D electrophoresis, also could be tried. Chromatographic conditions would have to be tested empirically so that sufficient separation and yield of the different species was achieved.

4.3 Sub-cellular distribution and cell surface exposure of PulA:CenB.

PulA:CenB was found in all sub-cellular fractions including the outer membrane of *K. oxytoca* (pMMBpulA:cenB) cells from cultures supplemented with maltose, for the induction of the PulA-specific secretory genes (Figure 23). However, the bulk of full-length PulA:CenB and its proteolytic products remained within the cytoplasm. About 8% of the total cell-associated PulA:CenB protein detected was located in the outer membrane. Immunofluorescence using intact cells (Figure 26) indicated that some PulA:CenB was accessible to the anti-CenB antibody, and therefore exposed to the extracellular medium.

PulA:CenB was not detected in periplasmic fractions of *K. oxytoca* (pMMBpulA:cenB) cultures grown in medium TYP supplemented with maltose (for expression of PulA-specific secretion genes). This supports the hypothesis of a trans-periplasmic channel through which PulA (and PulA:CenB) would reach the PulD channel in
the outer membrane (Pugsley and Possot 1993). If this hypothesis is correct, then PulA:CenB would be sequestered from soluble periplasmic proteins within a macromolecular structure and would not be isolated as part of the periplasmic fraction. Together with the CMCase assay results (Section 3.5) indicating a low level of extracellular cellulase activity, cell fractionation results suggest that secretion of PulA:CenB is impaired in some way (discussed further below).

Secretion of PulA:CenB appeared to interfere with secretion of PulA, as indicated by the reduction in surface exposed PulA activity from 40% to 12% of the total cell-associated activity (Table VI). These results suggest that PulA:CenB interacts with components required for the secretion of PulA from *K. oxytoca* cells. These components could be elements of either the Sec or PulA-specific pathways. By analogy to the mechanism of PulA secretion from *K. oxytoca* cells (see Figure 4), PulA:CenB would cross the inner membrane into the periplasm using the Sec proteins. Detection of PulA:CenB in association with the outer membrane suggests that an interaction with the PulA-specific secretory proteins is likely. However, this potential interaction did not result in PulA:CenB secretion to the extent observed for PulA secretion [based on the relative enzyme activities of surface-exposed PulA (40%) and PulA:CenB (6%)]. Whether the Sec- or PulA-catalysed steps are rate-limiting remains to be tested. Since it was not possible to induce expression of the PulA-specific secretory genes without also inducing *pulA* expression, I was unable to measure the efficiency of PulA:CenB secretion independently of PulA secretion. This could be done in an appropriate *pulA*\(^{-}\) strain of *K. oxytoca*, which could be created by replacement of the chromosomal *pulA* with a disrupted, cloned *pulA* gene.

In summary, not only is PulA:CenB predicted to interact with
proteins of the Sec pathway for translocation across the inner membrane [inferred from inner membrane and periplasmic localization of PulA:CenB (see Section 3.7)], but it is also predicted to interact with the proteins required for PulA-specific secretion. Therefore, once the components involved in intracellular blockage of PulA:CenB secretion have been identified, they might be modified by mutagenesis to increase levels of PulA:CenB secretion.


The altered growth kinetics between 4 and 10 hours after PulA:CenB induction of *K. oxytoca* (pMMBpulA:cenB) cultures grown in M63 medium indicated that PulA:CenB accumulation was interfering with cellular integrity. Since the presence of inclusion bodies was inferred from the analyses of strains *K. oxytoca* (pMMBcenB) and *K. oxytoca* (pMMBpulA:cenB) in TYP cultures induced with IPTG (Section 3.7.1), one interpretation of these results is that inclusion bodies decrease cell viability (culture growth) in the M63 but not the TYP medium. I attempted sub-cellular fractionation of cells from *K. oxytoca* (pMMBcenB) and *K. oxytoca* (pMMBpulA:cenB) cultures grown in M63 medium, but the cells from these cultures were too fragile to fractionate: osmotic shock resulted in complete cell lysis suggesting that cell envelope structures had been destabilized. Therefore, the cells grown in M63 medium under conditions in which CenB or PulA:CenB expression is induced are not as robust as those grown in TYP medium. It is interesting to note that *K. oxytoca* (pMMBpulA:cenB) cultures grown in M63 and induced with maltose underwent a more acute growth pattern change than cultures induced with IPTG, or than when *K. oxytoca* (pMMBcenB) was induced with IPTG (Figure 30; Since *cenB*-expressing *K. oxytoca* did not exhibit a dramatically modified growth pattern, it is likely that CenB inclusion bodies formed primarily within
the cytoplasm.). This observation was puzzling, considering that 4 times more CenB than PulA:CenB was produced (67 mg/ml versus 17 mg/ml), and CenB was formed in inclusion bodies compared to PulA:CenB when these cultures were grown in TYP and induced with IPTG. However, if CenB segments promote the formation of inclusion bodies in *K. oxytoca* cells, there could be two conflicting processes acting on PulA:CenB. The first (influenced by the CenB portion of the chimera) would result in the formation of inclusion bodies whereas the second (catalysed by the PulA portion of the chimera) would target the fusion protein for the secretory apparatus. Both processes may coincide such that some PulA:CenB proteins may aggregate while partially in transit across the inner membrane. Such a blockage of inner membrane translocation channels would have a detrimental effect on cell viability by preventing essential periplasmic components from leaving the cytoplasm.

Alternatively, both CenB and PulA:CenB may perturb the inner membrane in a manner similar to that described for a *phoE-lacZ* chimera that was expressed in *E. coli* (Tommassen *et al.* 1985). Expression of *phoE-lacZ* in *E. coli* was accompanied by a decrease in culture turbidity between 2 and 4 hours after induction of hybrid gene expression, as well as by induction of a 26 kDa protein, later identified as the cytoplasmic phage shock protein PspA (Brissette *et al.* 1990). It was subsequently observed that *pspA* induction occurred only when the Sec pathway was involved in translocation of the protein (or hybrid protein) in question (Kleerebezem and Tommassen 1993). Additionally, PspA was also detected in cultures in which fatty acid biosynthesis was inhibited by diazaborine (an inhibitor of enoyl-ACP reductase; Kater *et al.* 1994) or cerulenin (an inhibitor of β-ketoacyl-ACP synthase; Vance *et al.* 1972; White 1995), when signal peptidase II (LspA) was inhibited by globomycin [LspA removes signal peptides from lipoproteins (eg. PulA)
crossing the inner membrane; Bergler et al. 1994], and when defective forms of the outer membrane protein PulD accumulated in the periplasm (Hardie et al. 1996). It has also been shown that attempted translocation of PhoE mutants [containing various sequence modifications preventing inner membrane translocation (Kleerebezem and Tommassen 1993)] results in dissipation of the pH gradient across the inner membrane and induction of pspA expression (Kleerebezem et al. 1996). These observations suggest that proteins impaired in translocation across the inner membrane (such as was predicted for PhoE-LacZ; Tommassen et al. 1985) or lipoproteins that are not correctly processed somehow impair the pH gradient across the cytoplasmic membrane and thus inhibit membrane energization. Kleerebezem and colleagues have suggested that PspA re-establishes a proton gradient via an as yet uncharacterized mechanism, restoring cell viability (Kleerebezem et al. 1996).

I have shown that both CenB and PulA:CenB were associated with the inner membrane, and assume that they interact (via their respective signal peptides) with the Sec pathway. Since translocation via the Sec pathway requires ATP hydrolysis, attempted export of either CenB or PulA:CenB may overwhelm this pathway resulting in a collapse of the membrane potential in a manner similar to that proposed by Kleerebezem for the translocation of pre-PhoE (Kleerebezem et al. 1996). By analogy with PulA secretion (see Section 1.5.1), PulA:CenB should be processed by LspA before being acylated at the amino-terminal cysteine. PulA:CenB or CenB trapped within the inner membrane may dissipate ATP reserves, block LspA activity, or somehow inhibit fatty acid biosynthesis resulting in impaired growth (decline phase of growth curve in Figure 30) followed by expression of pspA which restores inner membrane energization (recovery phase in Figure 30). The identification of a PspA homologue accumulating in K. oxytoca (pMMBpulA:cenB) under conditions leading to altered growth patterns
would provide strong evidence for these hypotheses.

The "recovery" of membrane stability (*i.e.*, resumption of growth; Figure 30), manifested by the appearance of "survivors", may have been the result of secondary mutations (or induction of *pspA* expression) in the stressed cells. "Survivors" were thought to perhaps have enhanced extracellular cellulolytic activity and, therefore, $10^8$ "survivor" cells were spread onto M63 plates and inoculated into M63 liquid media containing CMC or avicel as the carbon source (identical to the media described in Sections 3.9.2 and 3.9.3). Although colonies were isolated on solid medium, no growth was obtained in liquid M63 media supplemented with CMC or avicel (similar to the results obtained in Sections 3.9.2 and 3.9.3). Therefore, although "survivor" cultures regained the ability to grow on M63 medium, it was not possible to isolate cultures able to grow on cellulose and therefore, the appearance of "survivors" was not associated with enhanced extracellular cellulolytic activity.

4.5 Growth of *K. oxytoca* (pMMBpulA:CenB) on cellulose.

*K. oxytoca* cultures supplemented with 0.1 mg/ml purified CenB grew in medium containing CMC or avicel as carbon source. This amount of purified CenB (0.1 mg/ml; 2.5 nmol/mg cellulose) added to cultures of *K. oxytoca* was *ca.* 6 fold greater than the amount of PulA:CenB that cultures of *K. oxytoca* (pMMBpulA:CenB) accumulated after 5 hours of induction with 0.1 mM IPTG (17 mg/L), and very little of this PulA:CenB (or the 100 kDa species predicted to be the CenB portion of the chimera) was secreted. The CMCase activity data (see Section 3.5) of *K. oxytoca* (pMMBpulA:CenB) cultures were interpreted to indicate about 6% (IPTG and maltose induced) or 16% (IPTG induced) of the total cell-associated activity was exposed to the extracellular
medium (see Section 4.2.1). This amount of surface-exposed CMCase activity would correspond to 1.0 - 2.6 mg/L PulA:CenB (based on the accumulation of 17 mg/ml PulA:CenB in these cultures under the conditions used), which is approximately 1/100th to 1/40th of the amount of the purified CenB protein added that resulted in almost one K. oxytoca culture doubling in 24 hours.

Therefore, it is not surprising that K. oxytoca (pMMBpulA:cenB) cultures did not grow on cellulose when expression of pulA:cenB was induced. Indeed, even if all the intracellular PulA:CenB (17 mg/L) were to be secreted this would still be 1/6th of the amount of purified CenB that resulted in growth when added to the cultures of K. oxytoca. However, since growing cultures would be continuously producing PulA:CenB in a ratio proportional to the number of cells in the culture, it is possible that if all the PulA:CenB produced were secreted, these cultures could grow on cellulose. However, it should be noted that expression of PulA:CenB resulted in membrane disruption. Although the fundamental biological mechanism(s) leading to this loss of cytoplasmic and periplasmic contents is not understood, this loss of cell integrity is a major obstacle to the long term goal of obtaining a strain that can degrade cellulose.

4.5.1 Mutagenesis of K. oxytoca (pMMBpulA:cenB) cultures.

Although it was not possible to obtain mutants of K. oxytoca (pMMBpulA:cenB) cultures that resulted in growth on cellulose in one round of mutagenesis (such mutations may be detrimental to the cell if they decrease recognition for other vital exported or secreted proteins, and therefore not detected by this strategy), it was considered possible to obtain a K. oxytoca (pMMBpulA:cenB) strain that has enhanced secretion of PulA:CenB, such that it grows on cellulose, by sequential
mutagenesis. *K. oxytoca* (pMMBpulA:cenB) treated with MNNG could be first screened for maltose-independent secretion of PulA (by assaying cell-surface pullulanase activity). If any promising mutants were isolated, successive rounds of mutagenesis might lead to mutant strains accumulating increased levels of PulA:CenB and expressing the PulA-specific secretory proteins in a maltose-independent manner.

Alternatively, specific interactions between PulA:CenB and each of the PulA-specific secretory proteins (*pulC-O/pulS*) could be tested. Cells from cultures of *K. oxytoca* (pMMBpulA:cenB), each with individual mutations in the secretory proteins (Pugsley *et al.*, 1990) could be fractionated and the intracellular location of PulA:CenB determined by Western blot. In this manner, the intracellular site of PulA:CenB blockage may be determined. Mutagenesis of the protein(s) involved in PulA:CenB blockage would then be tried and cells secreting enhanced levels of PulA:CenB selected by growth on cellulose.

4.6 Thio maltose analogues.

Analysis of 4 thio analogues of maltose indicated that analogue 4 was the most effective inducer of maltose regulon promoters. Although analogue 4 was neither as effective as maltose nor maltotriose, the finding that it was an inducer in *E. coli* pop3125 suggested that it was converted into a thio maltotriose analogue. Experiments conducted with analogue 4, in collaboration with Dr. W. Boos, may lead to the isolation of the putative enzyme maltose-maltotriose phosphorylase (MP; Decker *et al.* 1993) capable of converting analogue 4 into a triose analogue.
4.7 Future directions.

Complete evaluation of PulA as a determinant for the secretion of CenB from *K. oxytoca* cells was hampered by the expression of *pulA* and the PulA-specific secretory genes while maltose (maltotriose) was the inducer. Firstly, deletion of the chromosomal *pulA* (or at least most of the gene) would facilitate interpretation of PulA:CenB secretion data (and might lead to enhanced PulA:CenB secretion). Secondly, elimination of the need for maltose (or maltotriose) to express genes in the maltose regulon would facilitate interpretation of gene induction and cellulose growth experiments. Thirdly, it would be ideal for expression of the *pulC-O/pulS* (encoding PulA-specific secretory proteins) and *pulA:cenB* to occur simultaneously, and independently of genes involved in metabolism of other sugars. A non-metabolizable maltotriose analogue could be synthesized and used in laboratory experiments. However, in terms of the long term goal of industrial application of *K. oxytoca* secreting cellulases, large-scale use of a maltotriose analogue inducer would not be practical because of the high cost of synthesis and uncertain environmental consequences resulting from large scale use of this compound. Alternatively, a *K. oxytoca* strain expressing the MalT<sup>c</sup> allele (constitutively active MalT activator resulting in maltotriose independent expression of genes in the maltose regulon; Dardonville and Raibaud 1990) could be made and used. However, modification of the promoters required (or use of a constitutive promoter) for *pulA:cenB* and *pulC-O/pulS* expression would be more practical as expression of the maltose regulon would be separated from being expressed simultaneously with recombinant cellulases and the PulA-specific secretory genes.

Alternatively, one could make a variety of different fusion *pulA:cenB* genes encoding different amounts of the PulA sequence and
determine which (if any) produced a protein that was secreted into the medium with greater efficiency. These additional fusion genes would contain a portion of the cenB gene (encoding a catalytically active segment) and either only regions of pulA encoding segment A (the first 78 amino acids) and segment B (amino acids 735 to 814; Sauvonnet and Pugsley 1996), the entire mature protein-encoding portion of pulA, or portions of pulA between region B and the C-terminus.

Once a strain of K. oxytoca (pulA:cenB) with enhanced levels of PulA:CenB secretion was identified, analyses of PulA:CenB enzyme activity and membrane translocation could be done and the results compared to those described in this thesis. Such a comparison may increase our understanding of the processes and/or elements required for heterologous protein secretion.

If a strain of K. oxytoca secreting more than 0.1 mg/ml CenB was isolated, a second stage of experiments could be initiated. This second stage of experimentation would involve testing for growth on different types of cellulose (such as acid washed cellulose or wood waste from a pulp and paper mill) followed by testing for growth on cellulose without a source of fixed nitrogen. Once growth on cellulose under nitrogen fixing conditions was established, the use of this recombinant strain as a soil conditioner could be evaluated during a third stage of experiments. This could be done by inoculating an otherwise sterile cellulose-rich and nitrogen-poor propagation mixture of plant seedlings with the recombinant K. oxytoca strain, and comparing growth rates and survival of resultant plants to those of uninoculated seedlings.
The results described in this thesis are useful as part of the first stage of a larger project of creating a biological fertilizer from cellulose waste material. My evaluation of PulA as a tag for secretion of the PulA:CenB protein described indicates that PulA can catalyze secretion of enzymatically active CenB, albeit weakly and additional work is required to obtain efficient secretion of PulA:CenB from *K. oxytoca*.

As attempts toward the goal of obtaining efficient secretion of cellulases by *K. oxytoca*, several enzymatically active PulA:cellulase chimeric proteins were created. The PulA:CenB fusion protein produced in cells of *K. oxytoca* (pMMBpulA:cenB) was chosen for detailed study. PulA:CenB was maximally produced in response to the addition of 0.1 mM IPTG to cultures, such that maximum accumulation occurred after 5 hours of induction, and resulted in the accumulation of 17 mg/L PulA:CenB associated with intact cells and ca. 1.0 - 2.7 mg/L exposed to the extracellular medium. The full-length PulA:cenB was degraded into several smaller species, including predominant ones of 100 kDa and 47 kDa. The sizes of these proteolytic products, their ability to bind to avicel and immunoreactivity to anti-CenB antibodies, suggested that these species were CenB (100 kDa species) and CBD segments (47 kDa species) of PulA:CenB.

The full-length PulA:CenB and the 100 kDa proteolytic product had CMCase activity and some of this activity was detected on the surface of *K. oxytoca* cells. Additionally, PulA assays and cell fractionation data indicated that the PulA:CenB chimera interacted with both the Sec pathway (general pathway for translocation across the inner membrane) and the PulA-specific secretory proteins.
Although *K. oxytoca* cells grew using cellulose as a carbon source when supplemented with exogenous CenB, cultures of *K. oxytoca* (pMMBpulA:cenB) did not grow in cellulose-containing medium. MNNG mutagenesis of *K. oxytoca* (pMMBpulA:cenB) and selection on cellulose media also failed to isolate a strain capable of growth on cellulose. A step-by-step sequential mutagenesis protocol may be a better strategy for isolation of a multiply mutant strain of *K. oxytoca* (pMMBpulA:cenB) able to grow on cellulose.

Future studies on the strain *K. oxytoca* (pMMBpulA:cenB) to attempt maltose-independent expression of the PulA-specific secretion genes, as well as the construction of a more efficiently secreted PulA:CenB fusion protein, could result in a genetically engineered bacterium that can secrete sufficient amounts of cellulase allowing growth on cellulose.

Although it appeared that PulA was not an effective tag for secretion of CenB, this investigation has provided a number of intriguing avenues along which to proceed. In addition to the applied prospects, continuation of these investigations could lead to the isolation of a *K. oxytoca* homologue of PspA (required to re-establish a pH gradient across the inner membrane) and/or the proposed enzyme MP (involved in biosynthesis of maltotriose from maltose). Continuation of these experiments could also improve our understanding of the elements involved in native as well as heterologous protein secretion.
REFERENCES


Kornacker, M. G. and A. P. Pugsley (1990). The normally periplasmic enzyme β-lactamase is specifically and efficiently translocated through the Escherichia coli outer membrane when it is fused to the cell-surface enzyme pullulanase. Molec. Microbiol. 4: 1101-1109.


Sauvonnet, N., I. Poquet, and A. P. Pugsley (1995). Extracellular secretion of pullulanase is unaffected by minor sequence changes but is usually prevented by adding reporter proteins to its N- or C-terminal end. J. Bacteriol. 177: 5238-5246.


Gene (top strand) and deduced protein (bottom strand; single letter amino acid sequence indicated below each codon) sequence of the *C. fimi* CenA. Restriction sites used in subcloning are boxed and labelled. Signal peptide cleavage site is indicated by the arrow head. Pro-Thr rich spacer region is underlined. Sequence (and numbering) starts with the atg start codon and ends with the tga stop codon (indicated by an asterisk).

```
    atg tcc acc cgc aga acc gcc gca gcg ctg ctg gcc gcg gcc gcg 45
    M S T R R T A A A L L A A A A 15
    gtc gcc gtc ggc ggt ctg acc gcc ctc acc acc acc gcc gcg cag 90
    V A V G G L T A L T T T A A Q 30
    gcg gct ccc ggc tgc cgc gtc gac tac gcc gtc acc aac cag tgg 135
    A A P G C R V D Y A V T N Q W 45
    ccc ggc ggc ttc ggc gcc aac gtc aeg atc acc aac ctc ggc gac 180
    P G G F G A N V T I T N L G D 60
    ccc gtc tct gcg tgg aag ctc gac tgg acc tac acc gca ggc cag 225
    P V S S W K L D W T Y T A G Q 75
    Bam HI
    Pvu II
    ggg atc cag[boxed] cag ctg[boxed] tgg aac ggc acc gcg tcg acc aac ggc ggc 270
    R I Q Q L W N G T A S T N G G 90
    cag gtc tcc gtc acc agc ctg ccc tgg aac ggc agc atc ceg acc 315
    Q V S V T S L P W N G S I P T 105
    ggc ggc aeg gcg tct gtc tgg ttc gac gcc tcg tgg gcc ggc gcc tcg 360
    G G T A S F G F N G S W A G S 120
```
aac ccg acg ccg gcg tcg ttc tct tcg ctc aac ggc acc acc acc tgc acg
N P T P A S F S L N G T T C T 135

ggc acc gtc ccg acg acc acc acc ccc acc ccc acc ccc acc ccc acg acc
G T V P T T P P P P P P 150

cct ccc acg ccg acg ccg acc ccg acc ccc acc ccc acc ccc acg ccg acg
T P P P P P P P P 165

gtc acg ccg cag ccg acc acc ggc ttc tac gtc gac ccg acg acg
V T P Q P T S G F Y V D P T T 180

cag ggc tac cgc gcg tgc cag ggc gcg tgc ggc acg acg gac aag ggc
Q G Y R A W Q A A S G T D K A 195

cgt ctc gag aag atc gcc ctc gcc cag gcg tgc tac gtc ggc
L L E K I A L T P Q A Y W V G 210

aac tgg ggc gac gcg tgc cac gcg cag gcg gac gtc ggc gac gac tac
N W A D A H A Q A E V A D Y 225

cct ggc ccg gcc gtc gcg gcc gcg ggc gac ccg ccc cgc gtc gtc
t P T A V A A G K T P M L V V 240

tac gcg atc ccc gcg gcg gcc gac tgc gcc tgc cac tcc gcc ggt ggt 765
Y A I P G R D C G S H S C G G 255

gtc tcc gag tcc gag tac gcg cgc tgc gtc gac acc gtc gcg gac
t V S E E E Y A R W V D T V A Q 270

ggc atc aag ggc aac ccg atc gtc atc ctc gag ccc gac gcg ccc
t G I K G N P I V I L E P D A L 285

gcg cag ctc gcc gcc tgc gcc tcc gcc cag ggt gac gcc gcg gtc
c A Q L G D C S G Q G D R V G F 300

cct cag tac gcc gcc aag tgg ctc cct gcc ctc gcc gcg gcg
c L K Y A A T S L T L K G A R V 315

tac atc gac gcc gcc cac gcg aag tgg ctc gtc gac acc ccg
t Y I D A G H A K W L S V D T P 330

gtc aac cgc ctc aac cag gtc ggc ttc gag tac gcg gtc gtc
t V N R L N Q V G F E Y A V G F 345

gcg ctc aac acg tcc aac tac cag acg acg gcg gac gac aag ggc
t A L N T S N Y Q T T A D S K A 360
tac ggc cag cag atc tgc cag cgg ctg ggc ggc aag aag ttc gtc 1125
YGQQISQRLG-GKKFV 375
atc gac acc tgc cgc aac ggc aac ggc tgc aac ggc gag tgg tgc 1170
IDTSRNGNGSNGEGWCE 390
aac ccg cgc ggc cgc gcg etc ggc gaa cgc ccg gtc gcg gtg aac 1215
NPRGRALGERPVAVN 405
gac ggc tcc ggc ctg gac ggc etc ctg tgg gtc aag ctg ccc ggc 1260
DGSGL'DALLWVKLPG 420
gag tcc gac ggc gcg tgc aac ggc gcg gcg ggc gcg cag tgg 1305
ESDGACNGGPAAGQW 435
tgg cag gag atc gcc ctg gag atg gcg cgc aac ggc agg tgg tga 1350
WQEIALERMANARNWR 449
7.2  cex/Cex sequences.

Gene (top strand) and deduced protein (bottom strand; single letter amino acid sequence indicated below each codon) sequence of the *C. fimi* Cex. Restriction sites used in subcloning are boxed and labelled. Signal peptide cleavage site is indicated by the arrow head. Pro-Thr rich spacer region is underlined. Sequence (and numbering) starts with the atg start codon and ends with the tga stop codon (indicated by an asterisk).
ggc cac acg ctc gta tgg cac tcg cag ctg ccc gac tgg gcg aag 405
G H T L V W H S Q L P D W A K 135
aac ctc aac ggc tcc gcg**ttc gag agc gcg atg gtc aac cac gtg 450
N L N G S A F E S A M V N H V 150
acg aag gtc gcc gac cac ttc gag ggc aag gtc gcg tcc tgg gag 495
T K V A D H F E G K V A S W D 165
gtc gtc aac gag gcg ttc gcc cac ttc ggc gcg ggc ggc gcg cgg cag gac 540
V V N E A F A D G G R R Q D 180
tcg gcg ttc cag cag aag ctc gcc aac gcc tac atc gag acc gcg 585
S A F Q Q K L G N G Y I E T A 195
ttc cgg gcg gca cgt gcg gcg gac ceg acc gcc aag ctc tgc atc 630
F R A A R A A D P T A K L C I 210
aac gac tac aac gtc gag ggc atc aac gcg aag agc aac tcc ctc 675
N D Y N V E G I N A K S N S L 225
tac gac ctc gtc aag gac ttc aag gcg cgc gcc gtc ccc ctc gcc 720
Y D L V K D F E K A R G V P L D 240
tgc gtc ggg ttc cag tcc cac ctc atc gtc gcc cag cgg ggc 765
C V G F Q S H L I V G Q V P G 255
gac ttc cgg cag aac ctc cag cgg ttc gcg gac ctc ggc gtg gcg 810
D F R Q N L Q R F A D L G V D 270
gtg gcg atc acc gag ctc gcc atc cgg ctc atg aeg gcc ccc ctc gcc 855
V R I T E L D I R M R T P S D 285
gcg acc aag ctc gcg acc cag ggc gcg gcc tac aag aag gtc gtg 900
A T K L A T Q A A D . Y K K V V 300
cag gcc tgc atg cag gtg acc cgc tgc cag ggc gtg acc gtc tgg 945
Q A C M Q V T R C Q G V T V W 315
ggc atc acc gcc aag tac tcc tgg gtg gcg gcc gtc ttc ccc gcg 990
G I T D K Y S W V P D V F P G 330
gag ggg gcc gcg ctc gtg tgg gag gcg gcg acc tac gcc aag aag ceg 1035
E G A A L V W D A S Y A K K P 345
gcc tac gcc gcc gtg atg gag gcc ttc ggc gcg agc cgg acg cgg 1080
A Y A A V M E A F G A S * P T P 360
acg ccc acc acg ccc acc ccc' acg acg ccc acg ccc acc 1125
T P T T P T P T T P T T P T 375

ccg acg tcc ggt ccc gcc ggg tgc cag gtg ctc tgg gcc gtc acc 1170
P T S G P A G C Q V L W G V N 390

cag tgg aac acc gcc ttc acc gcc aac gcc gtc aac gcc aac acg 1215
Q W N T G F T A N V T V K N T 405

tcc tcc gct ccc gcc gtc gac gcc tgg acg ctc acc gcc gcc aac tcc 1260
S S A P V D G W T L T F S F P 420

tcc ggc cag cag gcc tcc acc gcc cag gcc tgg acc gcc gcc gcc 1305
S G Q Q V T Q A W S S T V T Q 435

tcc ggc teg gcc gtc aag gcc gcc gcc tgg aac gcc ccc ggc teg 1350
S G S A V T V R N A P W N G S 450

atc ccc gcc gcc gcc acc gcc cag tcc gcc tcc gcc gcc gcc teg cac 1395
I P A G G T A Q F G F N G S H 465

acg gcc acc aac gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc 1440
T G T N A A P T A F S L N G T 480

ccc tgc acg gtc ggc tga 1458
P C T V G * 485
7.3 \textit{cenB/CenB} sequences.

Gene (top strand) and deduced protein (bottom strand; single letter amino acid sequence indicated below each codon) sequence of the \textit{C. fimi} CenB. Restriction sites used in subcloning are boxed and labelled. Signal peptide cleavage site is indicated by the arrow head. Pro-Thr rich spacer regions are underlined. Sequence (and numbering) starts with the atg start codon and ends with the tga stop codon (indicated by an asterisk).

\[
\begin{align*}
\text{atg ctc cgc cca gtc cca cgc aag ctc gtc gcg ggt ggc tcc gcc} & \text{ 45} \\
\text{MLRQVPRTLVALGGSAS} & \text{ 15}
\end{align*}
\]

\[
\begin{align*}
\text{ctc ggc gtc gcc gtc ggg gtc gtc gtc ggc cct ggc acc ggc} & \text{ 90} \\
\text{LAVAVGVVLVAPLALTG} & \text{ 30}
\end{align*}
\]

\text{Not I} \downarrow
\[
\begin{align*}
\text{ggg gag gcc ccc acc tac aac tac gcc gag gcc ctg cag aag} & \text{ 135} \\
\text{AAPTYNYAEALQK} & \text{ 45}
\end{align*}
\]

\[
\begin{align*}
\text{tgg atg ttc ttc tac cag ggc cag cgc tcc ggc gac ctg ccc gcc} & \text{ 180} \\
\text{SMFQYQAQRSGDLP} & \text{ 60}
\end{align*}
\]

\[
\begin{align*}
\text{gac ttc cgc gtc tcc tgg cgc ggc gac tcc ggc gac ctg acc gac ggc} & \text{ 225} \\
\text{DFPSWRGDSGLTDG} & \text{ 75}
\end{align*}
\]

\[
\begin{align*}
\text{ggc gac gtc ggc aag gac ctc acc ggc ggc tgg tac gac gcc ggc} & \text{ 270} \\
\text{ADVGLTGGYDAG} & \text{ 90}
\end{align*}
\]

\[
\begin{align*}
\text{gac cac gtg aag ttc ggc ttc cgc atg gcg ttc agc gcc aag atg} & \text{ 315} \\
\text{DHVKFGFMPMAFSATM} & \text{ 105}
\end{align*}
\]

\[
\begin{align*}
\text{ctc ggc tgg ggc ggc atc gag agc ccc aag ggc tac tcc aag ggc} & \text{ 360} \\
\text{LAWGAIESTGYP} & \text{ 120}
\end{align*}
\]

\[
\begin{align*}
\text{ggc tgc ctc gac gag ctc aag gac aac ctg cgg ttc gtc agc gac} & \text{ 405} \\
\text{GSLDELKDNLRFTSV} & \text{ 135}
\end{align*}
\]
tac ttc gtc aag ggc cac act gcc ccg aac gag ctc gtc tac gtg cag 450
Y F V K A H T A P N E L Y V Q 150
gtc ggc gac ggc gag ggc gac cac aag tgg tgg gga ccc gcc gag 495
V G D G E A D H K W W G P A E 165
gtc atg acc atg ggc cgg ccc tgc cac aag atc agc ggc tcc tgc 540
V M T M A R P S H K I S A S C 180
ccc ggc tgc gac tgc cgc ggc gag acg gcc ggc cgg ctc ggc tgg 585
P G S D V A A E T A A A L A S 195
tcg ggc atc tgc tgc aag ggc gac ggc ccc ggc tac ggc ggg acc 630
S A I V L K G D D P A Y A A T 210
ctc gtc tgg cac gcc aag cag ctc tac acg tcc ggc gac acc tac 675
L V S H A K Q L Y T F A D T Y 225
cgc ggc ggc tac gcc gtc gcc ggc gcc cgg ctc ggc tac tac 720
R G A Y S D C V T A A S A Y Y 240
aag tcc tgg tcc ggc tac cag gac gag ctc gtc tgg ggc ggc tac 765
K S W S G Y Q D E L V W G A Y 255
tgg ctc tac aag gcc acc ggt gac ggc acg acg tac ctc gcc aag gcc 810
W L Y K A T G D A T Y L A K A 270
gag gcc gag tac gcc aag ctc gcc acg gag aac cag aac gcc acc acg 855
E A E Y D K L G T E N Q S T 285
cgc tcc tac aag tgg acg atc ggc tgg gac aac aag cag ttc ggc 900
R S Y K W T I A W D N K Q F G 300
acg tac ggc ctc gtt ctc gcc atg gag acc gcc aag cag aag tac gtc 945
T Y A L L A M E T G K Q K Y V 315
gac gcc ggc aac cgc tgg ctc gac tac tgg acc gcc gtc ggc gtc aac 990
D D A N R W L D Y W T V G V N 330
ggc cag aag gtt ccc tac tcc cgg gga ggc cag gcc gtc ctc gac 1035
G Q K V P Y S P G G Q A V L D 345
tcg tgg ggt gcc cgg cgg tac gcc gcc aac gcc acc cgg gtc ttc gtc 1080
S W G A L R Y A A N T S F V A 360
ctc gtc tac tcc gac tgg atg acc gcc ggc acc cgc aag gcc cgg 1125
L V Y S D W M T D A T R K A R 375
7.4 *pulA/PulA* sequences.

Gene (top strand) and deduced protein (bottom strand; single letter amino acid sequence indicated below each codon) sequence of the *K. oxytoca* *pulA*. Restriction sites used in subcloning are boxed and labelled. Signal peptide cleavage-site is indicated by the arrow head. Regions A (double underline) and B (dashed underline) essential for PulA secretion are indicated in the sequence. Sequence starts with the atg start codon and ends with the tga stop codon (indicated by an asterisk). Modifications introduced by PCR mutagenesis are indicated above the appropriate sequences (starting at nucleotide 2501).
ggc gac ttc acc gat cgt acg gta tcg gtg att gcc ggt aac age 495
G D F T D R T V S V I A G N S 165
gcg gtc tat gac tcc cgc gcc gac gcc ttc cgc gec gct ttt ggc 540
A V Y D S R A D A F R A A F G 180
gtc gcg ctg gcc gaa gcc cac tgg gtc gac aaa aat acg ctg ctg 585
V A L A E H W V D K N T L L 195
tgg ccg ggc ggg cag gat aag ccc atc gtt ggc ctc tac tac gac 630
W P G G Q D K P I V R L Y Y S 210
cac agc agt aaa gtc gcg gcc gac ggc gaa gcc aaa ttt acc gac 675
H S S K V A A D G E G K F T D 225
cgc tac ctg aag ctg agc ccg act acc gtc agc cag cag gtc gac 720
R Y L K L T P T T V S Q Q V S 240
atg cgc ttc ccg cac ctt tcc agc tat gcg gcc ttt aag ctg ccc 765
M R F P H L S Y A A F K L P 255
gac aac gcc aac gtg gac gag ctg ctg cag ggc gaa acg gtc gcc 810
D N A N V D E L L Q G E T V A 270
att gcc gcc gca gag gac ggg atc ctg att tcc gcc acc cag gtc 855
I A A A E D C I L I S A T Q V 285
cag acg gcg gcc gtc ctg gat gac gcc tat gcc gaa ggc gcg gaa 900
Q T A G V L D D A Y A E A A E 300
gcc ctg agc tac ggc gcg cac ctg gcc gac ggc gcc gtc acc ttc 945
A L S Y G A Q L A D G G V T F 315
cgc gtt tgg gcg ccg act gcc cag cag gtg gat gtt gtt gtc tac 990
R V W A P T A Q Q V D V V V Y 330
agc gcc gat aag aaa gtc att ggc agc cat ccg atg acc cgc gcc 1035
S A D K K V I G S H P M T R D 345
agc gct tcc ggc gcg tgg tca tgg cag ggc gcc agc gat ctg aad 1080
S A S G A W S W Q G G S D L K 360
ggc gcg ttc tac cgc tat gcc atg acg gtt tat cac ccg cag tcc 1125
G A F Y R Y A M T V Y H P Q S 375
cgt aag gtt gaa cag tac gaa gtc acc gcc cag ctg tat gcc cat aag 1170
R K V E Q Y E V T D P Y A H S 390
Bel I

gac aac cag acg ctg tgg gac \text{agt atc} aac tac aaa gcg tcc cag 2565
DNQTLWDMI SYKASQ 855
gaa gcc gat ctg gcg acc cgc gtt cgc atg cag gcc gtt tcc ctg ctg 2610
EADLATRVRMQAVSL 870
gcg acg gtg atg ctg ggg cag ggg atc gcg ttc gat cag cag ggc 2655
ATVMLGQGIAFDQQG 885
tct gaa ctg ctg cgt tcc aaa tcc ttt acc cgc gac tcc tac gac 2700
SELRSKFSTRDSYD 900
tcc ggc gac tgg ttc aac cgc gtg gat tat tcc ctg cag gat gtt cag gat aat 2745
SGDWFNRYSLQDN 915
aac tac aac gtt gct cgc atg cgc gtt gtt gcg agar aac cgc gac ggc 2790
NYNVPRLSDGSS930
tat gag gtg att act cgc gtc aaa gag atg gtt gcc acc cgc ggc 2835
YEVTIRKEMVATPG 945
gaa gcg gag ctg aag cag atg acc gcg ttc tac cag gac tgg gtt cag 2880
E AE L K Q M T A F Y Q E L T 960
gag ctg cgg aaa tcc ctc ccc ctt ggc gat ggc agc 2925
ELRKSSTPLFGLDGS 975
gcg gta atg aag cgc gtt gtt ttc ctc aat acc cgg gat tcc gac cag 2970
A V M K R V D F R N T G S D Q 990
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Q A G L L V M T V D D G M K A 1005
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GASLDSDGLDGLVVIA 1020
aac gcc gcg cca ggg gaa acg cgc ccg cgg gcg ctt gcc gat ggg aat ggg aag 3105
NAAPESRTELNFAG 1035
acg ctg cag cag acg gcc acg gcc ctg cag cag acg ggc gaa aac tcc 3150
TLQQLSAPAIAQQTAGENS 1050
tgt gcc cgg gcc gcc tgg gcc ctg att ggc gct gat gcc acc gcc act cag 3195
LANGVQIAADGTVTL 1065
ccg gcc ggg tgg ctc gcc gct ggg cgc ggt gtt gcc ctg gaa ctg cag ggc aag 3240
P A W S V A V L L P Q G E A 1080
cag gcc gcc ggg cgg cgc gta agt aag taa 3273
QGALPVSNSK* 1090