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Gram-scale synthesis of recombinant chitooligosaccharides in *Escherichia coli*

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Abstract

Cultivation of *Escherichia coli* harbouring heterologous genes of oligosaccharide synthesis is presented as a new method for preparing large quantities of high-value oligosaccharides. To test the feasibility of this method, we successfully produced in high yield (up to 2.5 g/L) penta-*N*-acetyl-chitopentaose (**1**) and its deacetylated derivative tetra-*N*-acetyl-chitopentaose (**2**) by cultivating at high density cells of *E. coli* expressing *nodC* or *nodBC* genes (*nodC* and *nodB* encode for chitooligosaccharide synthase and chitooligosaccharide *N*-deacetylase, respectively). These two products were easily purified by charcoal adsorption and ion-exchange chromatography. One important application of compound **2** could be its utilisation as a precursor for the preparation of synthetic nodulation factors by chemical acylation. © 1997 Elsevier Science Ltd.

Keywords: Nod genes; Chitooligosaccharides; Recombinant oligosaccharides; *N*-deacetylation; High cell density culture

1. Introduction

It is now well established that both free and conjugate oligosaccharides play a key role in immunological and biochemical recognition. As oligosaccharides are usually obtained in fairly low yield by purification from natural sources, the synthesis of oligosaccharides has thus become a major challenge in carbohydrate chemistry to provide sufficient amounts of well-characterised oligosaccharides needed for fundamental research and potential applications [1].

Although chemical methods for the synthesis of oligosaccharides have considerably improved during the past twenty years, they still need many protection and deprotection steps. As the number of steps increases with the size of the oligosaccharide, preparation of large quantities of oligosaccharide longer than trisaccharide is not practical.

Enzymatic methods are becoming more popular because they allow regioselective synthesis under mild conditions and without protection of the hydroxyl groups [2–5]. Depending on the donor molecule, one can distinguish several types of enzymes used in in vitro oligosaccharide synthesis. Glycosyl hydrolases and glycosyl phosphorylases act physiologically as depolymerising enzymes but can

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be used in synthesis by controlling the equilibrium or the kinetics of the reaction. The substrate and enzymes are easily available, but these reactions are not very versatile. Glycosyl transferases from the Leloir pathway display high regiospecificity for the acceptor and the donor substrate. Glycosyl transferases are not as readily available as glycosyl hydrolases, but a number of them have been cloned recently and could thus be produced as recombinant enzymes. The major drawback in the utilisation of glycosyl transferases is the availability and the prohibitive cost of the sugar nucleotides used as activated sugar donors, even if this cost can be significantly reduced by an in situ regeneration of the sugar nucleotides. Our idea was to use growing bacterial cells as natural mini-reactors for the regeneration of sugar nucleotides and to utilise the intracellular pool of sugar nucleotide as substrate for the in vivo synthesis of 'recombinant' oligosaccharides by recombinant glycosyl transferases. To establish the feasibility of this new method of oligosaccharide synthesis, we attempted to prepare recombinant chitooligosaccharides (COs) for the following reasons:

(1) chitin oligomers are very difficult to prepare in high yield by either chitin hydrolysis [6] or chemical synthesis [7];

(2) they are of great biological interest since free chitooligosaccharides have been shown to elicit biological responses in plants [8]. In addition, chitopentamer and chitotetramer form the backbone of substituted lipochitooligosaccharides [9–12] which induce nodulation in leguminous plants, thus representing a unique class of plant growth regulator;

(3) the biosynthetic pathway of these lipochitooligosaccharides has been described recently, making available the genes involved in the synthesis of modified chitooligosaccharides [13–17]. The first committed step is the formation of the chitooligosaccharide core and is catalysed by NodC [18,19]. The resulting oligosaccharide is then *N*-deacetylated by NodB prior to the introduction of species-specific substitutions and the attachment of an acyl chain on the free amino group of the non-reducing end terminal glucosaminyl residue;

(4) UDP-*N*-acetylglucosamine, which is the sugar donor for the synthesis of *N*-acetylated chitooligosaccharide by NodC [18], is also the precursor for the biosynthesis of peptidoglycan and is therefore normally maintained at quite high intracellular concentration in growing bacterial cells [20,21].

In this paper, we report that the high cell density cultivation of *Escherichia coli* cells harbouring the *nodC* gene is a very efficient way to synthesise chitopentaose with a yield of 2.5 g per litre of culture medium. In addition, we show that cells also harbouring the gene *nodB* produced mono-deacetylated chitopentaose, and that the yield of deacetylation can be increased by engineering *nodB*. We also describe a simple and efficient two-step procedure to purify these recombinant oligosaccharides from cells.

2. Results

CO production in batch culture.—In order to determine whether COs could be effectively synthesised at the preparative scale in recombinant bacteria, we first developed a method to quantify the CO concentration in *E. coli* strains expressing *nodBC* genes. Using an assay based on a simple colorimetric determination of osamine content in cell extract after acid hydrolysis, we found that a batch culture of strain DH1 (pUCNBC) on LB medium produced ~ 10 mg per litre of COs. As some component of the LB medium interfered with the colorimetric determination of osamines, and complex media in general are not suitable for high cell density cultivation, bacterial cells were grown on mineral medium. CO yields were found to be higher with glycerol instead of

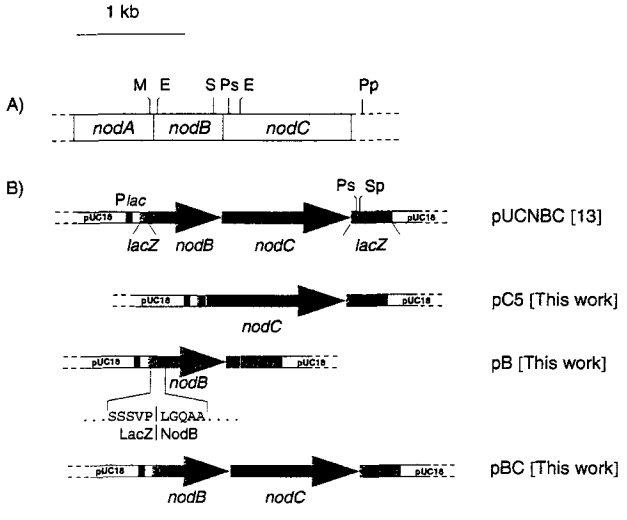

Fig. 1. Genomic organisation of azorhizobial *nod* genes. A) Restriction map of the relevant genes from *A. caulinodans* Nod locus *I* [25]. B) Plasmids used on this study (references between brackets). Plasmids pC5, pB, and pBC were constructed as indicated in the Experimental. A fusion protein between LacZ and NodB was constructed in pB and pBC. The peptide sequence of the region around the fusion is shown in pB. M, *Msc*I; E, *Eco*130I; Ps, *Pst*I; Pp, *Ppu*MI; S, *Sac*I; and Sp, *Sph*I.

glucose as the carbon source. Since in pUCNBC *nodBC* genes are under the control of the *lac* promoter (Fig. 1), we investigated the influence of the inducer isopropylthio-β-D-galactopyranoside (IPTG) on CO production by two *E. coli* strains, JM101 and DH1, producing different levels of the *lac* repressor. The strain JM101 (pUCNBC) which overproduces the *lac* repressor had a lower CO yield in absence of IPTG. By contrast, in strain DH1 the omission of IPTG did not reduce CO production. From these results, strain DH1 was selected as producer strain, and all cultures employed glycerol medium without IPTG.

High cell density cultivation.—To overcome problems of oxygen supply, nutrient availability, and accumulation of toxic by-products that arise in growing *E. coli* to high cell density [22–24], we used here a simple fed-batch technique with a constant glycerol feeding of 4.5 g/L · h. With the control strain DH1, this strategy allows a linear increase in biomass up to an OD of 150 (Fig. 2A). On the contrary, with strain DH1 (pUCNBC) a severe growth inhibition was observed after 5 h of feeding. This inhibition was overcome by lowering the glycerol feeding rate down to 2.4 g/L · h when the optical cell density of the culture reached the value of 50 (Fig. 2B). In these conditions, the growth proceeded linearly over a period of more than 24 h at a lower rate but without any inhibition. In addition, as COs were still produced at the same rate, the specific cellular concentration of CO increased progressively, almost doubling within 16 h of cultivation at a low feeding rate. After 48 h of cultivation, the final CO yield was higher than 1 g per litre of culture medium. This two-phase fed-batch cultivation strategy was used for further experiments of CO production.

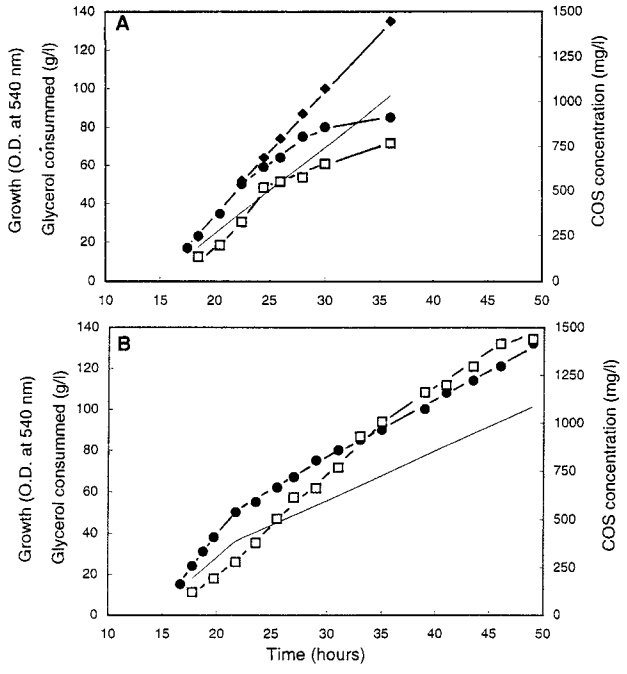

Fig. 2. High cell density fed-batch cultures at high (**A**) and low (**B**) feeding rate: (●) growth of strain DH1 (pUCNBC), (♦) growth of plasmid-free strain DH1, (□) CO production. The line represents the cumulated concentration of added glycerol. COs were quantified using purified compound **2** as standard.

Purification and identification of COs.—After centrifugation of the culture broth, COs were recovered exclusively in the pellet containing the bacterial cells. After disruption of the cells by boiling, cell debris was removed by centrifugation and the COs were purified by adsorption on activated charcoal as shown in Table 1. HPLC analysis of the CO fraction eluted with 30% ethanol showed two main peaks. Peak I had the same retention time as that of an authentic standard of penta-*N*-acetyl-chitopentaose (**1**), and peak II was further shown to be tetra-*N*-acetyl-chitopentaose (**2**). These two pentasaccharides

Table 1
Purification of chitooligosaccharide from 1 L of high cell density culture of strain DH1 (pUCNBC)

Purification steps	Volume (mL)	Chitooligosaccharides concn (mg/mL)	Yield (%)
Boiled cells supernatant	800	1.4	100
Charcoal purification			
- Non-adsorbed	1000	0	0
- 10% Ethanol	1000	0.09	7.8
- 30% Ethanol	1000	0.72	62
- 40% Ethanol	1000	0.16	14
Dowex 50 WX4			
- Flowthrough fraction	1000	0.22	19
- 2% aqueous NH_3	33	11.8	34

were separated by ion-exchange chromatography on Dowex 50 WX4 (Table 1). The flowthrough fraction contained compound **1**, while compound **2** was eluted with 2% aqueous NH_3. After removal of NH_3, **2** was shown to be 90% pure by HPLC and a typical yield of 400 mg(dry weight) per litre of culture medium was obtained.

NMR and mass spectral data confirmed the identification of compound **1** as penta-*N*-acetyl-chitopentaose [6]. The structure of compound **2** was clearly deduced from its ^1H NMR, ^{13}C NMR, and MS spectra. The ^1H NMR spectrum (Fig. 3) shows three types of signals in the H-1 region: the reducing H-1^1 at δ 5.07 ($J_{1,2}$ 2.0 Hz) and 4.57 ($J_{1,2}$ 7.0 Hz); interglycosidic H-$1^{2,3,4}$ at δ 4.48, 4.46, and 4.45 ($J_{1,2}$ 8.0 Hz); and the non-reducing end H-1^5 at δ 4.36 ($J_{1,2}$ 8.0 Hz). A high field signal at δ 2.6 (H-2^5) together with two acetyl signals at 1.95 and 1.92 (12H) are also present. The pseudo-molecular ion at *m/z* 992 ($[M + H]^+$) under the FAB$^+$MS technique and two ions at *m/z* 162 and 830 under the MIKE mode (data not shown) confirm the given structure.

Improvement in production of penta-N-acetyl-chitopentaose (**1**).—To obtain pure compound **1**, we subcloned the SacI-PaeI fragment from pUCNBC comprising *nodC* into SacI-PaeI-digested pUC18 (Fig. 1). Transformants harbouring the resulting plasmid pC5 grew very poorly at 37 °C and must be cultivated below 34 °C. High cell density culture of strain DH1 (pC5) resulted in a final CO yield of 2.5 g/L. This yield was considerably higher than those obtained with strain DH1 harbouring pUCNBC. After purification on activated charcoal, **1** was the major chitooligosaccharide detected together with a trace (less than 2%) of chitotetraose, and a final yield of 2.1 g(dry weight) per litre of culture was obtained.

Improvement in production of tetra-N-acetyl-chitopentaose (**2**).—HPLC analysis of charcoal eluates showed that compound **2** represented about 64% of the total chitopentamers produced by strain DHI (pUCNBC) at the end of the fermentation. HPLC analysis also showed that the yield of deacetylation was slightly lower (55%) after only 24 h of cultivation. Attempts were then made to improve the deacetylation yield by extending the cultivation time with different feeding strategies and culture conditions. However, no significant increase in the ratio **2**/**1** could be observed in any of the cases.

In *A. caulinodans*, *nodB* was shown to be preceded by a Shine Dalgarno sequence located at a non-optimal position [25]. This could result in an insufficient production of deacetylase activity, explaining the uncompleted deacetylation of **1** to **2**. We tried to increase the expression of *nodB* by removing

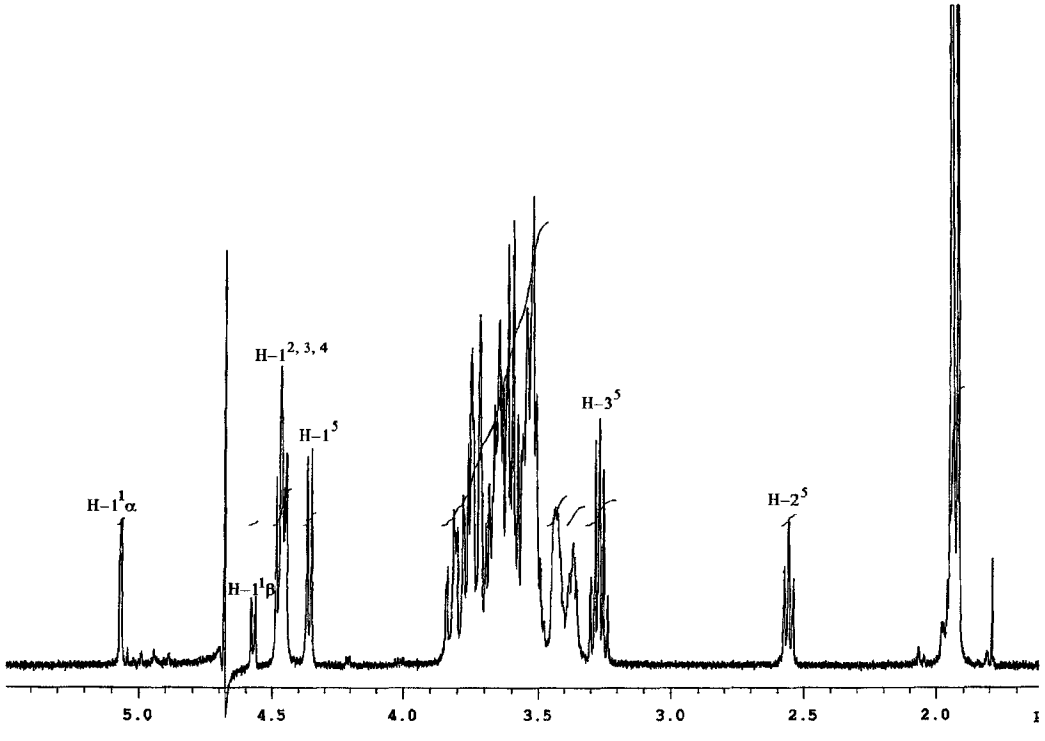

Fig. 3. ^1H NMR spectrum of tetra-*N*-acetyl-chitopentaose (**2**).

in pUCNBC the 200 bp fragment located between *nodB* and *lacZ'* in such a way that NodB would be expressed as a fusion protein with LacZ' (Fig. 1). HPLC quantification of **2** and **1** produced by strain DH1 harbouring the resulting plasmid pBC showed a significant increase in the deacetylation yields which reached the values of 64 and 82% after 24 and 40 h of cultivation, respectively. Furthermore, the total COs yield was also slightly improved throughout the fermentation time course and, after purification of compound **2** on charcoal and on Dowex 50 WX4, a final yield of 550 mg per litre of culture was obtained.

3. Discussion

The basic strategy for optimising recombinant protein production consists of overexpressing their genes. In the case of recombinant oligosaccharide production, the goal is not to produce a maximal yield of enzyme but to obtain a sufficient amount of active enzyme to catalyse the oligosaccharide synthesis at a maximal rate without disturbing cell metabolism. Our results show that the induction of the *lac* promoter by IPTG, which normally increase the expression of the *nodC* gene, did not improve the CO production. This suggests that a low expression level of *nodC* is enough to allow a maximal CO production. In addition, gene overexpression could be unfavourable to oligosaccharide synthesis by causing an unnecessary metabolic burning, a growth inhibition by overproduced proteins [26], and problems of precipitation and misfolding of enzymes [27].

We found that recombinant CO can account for up to 5% of the total cell dry weight. Since the peptidoglycan components derived from UDP-GlcNAc represent only 1.2% of the cell dry weight of a normal *E. coli* cell [28], the UDP-GlcNAc flow must have undergone a five-time increase. In growing bacterial cells, the pool level UDP-GlcNAc has been estimated to be 125 μM [20]. As the K_m value for UDP-GlcNAc of *Rhizobium freedi* NodC was shown to be only 42 μM [29], the UDP-GlcNAc concentration should not limit CO synthesis if the cellular machinery has the enzymatic and energetic capacity to maintain the UDP-GlcNAc pool at its physiological state. Although it is difficult to answer this question without experimentally determining the UDP-GlcNAc pool level in our culture, one can argue in favour of a non-limitation of chitooligosaccharide synthesis by UDP-GlcNAc concentration: (1) the enzymes involved in UDP-GlcNAc synthesis have been shown to be in relative excess [21]; (2) a depletion of the UDP-GlcNAc pool would probably result in cell lysis; (3) whereas NodC protein was shown to produce a mixture of trimer, tetramer, and pentamer at low UDP-GlcNAc concentration and pentamer at high UDP-GlcNAc concentration [29], we have obtained only chitopentamers; (4) high cell density culture was performed at low growth rate with consequently a low UDP-GlcNAc requirement for cell wall biosynthesis. However, in the case where it could be demonstrated that the rate of oligosaccharide synthesis is limited by the regeneration of the sugar nucleotide, a metabolic engineering of the biosynthesis pathway of the sugar donor could be considered.

Recombinant chitooligosacharides are produced intracellularly, and their cytoplasmic concentrations in culture having the highest CO yield can be roughly estimated at more than 15 g/L. Chitopentamers are still soluble at this concentration. One important result of this study is that such high concentration of foreign oligosaccharides apparently do not affect the growth and the cellular metabolism of the bacteria. However, it is not known whether a high CO concentration can inhibit the activity of NodC. By this hypothesis, compound **2** may have a more pronounced inhibitory effect than compound **1**. This difference would explain the considerably lower CO yield in culture producing both **2** and **1** as compared with culture producing only **1**.

NodC belongs to the same family of polysaccharide synthases as chitin, cellulose, and hyaluronate synthases [30]. In these enzymes, the mechanism of elongation is believed to proceed by a successive addition of new sugar residues to the growing acceptor chain. This chain is supposed to slide in the enzyme to leave the sugar donor sites free and to allow the reaction to continue. This mechanism results thus in the formation of long chain polysaccharides. In contrast, NodC was shown to only synthesise oligomers of 2–5 saccharide residues. Surprisingly, in our production process, we found that NodC almost exclusively directs the synthesis of pentasaccharides. It is known that UDP-GlcNAc to a certain extent controls the degree of polymerisation by favouring at low concentration the synthesis of chitobiose and chitotriose [29]. In our system, the exclusive formation of pentamer is probably due to the fact that the synthesis was carried out in growing *E. coli* cells in which the physiological pool of UDP-GlcNAc is maintained at a high level.

One important application of this method of CO synthesis is the utilisation of compound **2** as precursor for the preparation of synthetic nodulation factors by chemical acylation of the free amino group of the non-reducing end terminal residue. Substitution on the chitosaccharide backbone of the nodulation factor has been shown to determine the host specificity and biological activity [10]. It will thus be of great interest to produce in vivo the substituted chitosaccharide backbone by coexpressing the gene involved in the introduction of the substitutions. On the other hand, the length of the backbone seems to be species-specific and utilisation of *nodC* genes from strains which naturally secrete LCO tetramer may allow the in vivo production of chitotetramers.

The recent finding that the *Xenopus laevi* developmental protein DG42 is able to synthesise COs suggest that molecules related to COs may be involved in signalling during vertebrate development [31,32]. If the DG42 gene can be successfully expressed in *E. coli*, our CO production system could be used to synthesise and identify these signalling molecules. Other applications we can envision are the synthesis of substrates and inhibitors of chitinases or the preparation of chitooligosaccharide supports for affinity chromatography.

To our knowledge, our work is the first example of a gram-scale production of 'recombinant' oligosaccharides, and it demonstrates the possibility of using recombinant technology for the production of high-value oligosaccharides.

4. Experimental

Bacterial strains, plasmid and growth conditions.—*Escherichia coli* DH1 (DSM 4235) and JM101 (DSM 3948) were obtained from the Deutsche Sammlung von Mikroorganismen. Plasmid pUCNBC [13] was kindly provided by Peter Mergaert (University of Ghent); plasmid pUC18 was purchased from Pharmacia. Routine cultures of *E. coli* were performed in LB medium [33]. Mineral medium (MM) had the following composition: $(NH_4)_2HPO_4$ (4 g/L), KH_2PO_4 (10 g/L), $MgSO_4 \cdot 7H_2O$ (1 g/L), thiamine (4.5 mg/L), ampicillin (50 mg/L), trace mineral soln (10 mL/L); pH was adjusted to 6.8 with 5 N NaOH; $MgSO_4$ was autoclaved separately. Ampicillin and thiamine were sterilised by filtration. The trace mineral stock soln contained: nitrilotriacetate (70 mM, pH 6.5), $FeSO_4 \cdot 7H_2O$ (5 g/L), $MnCl_2 \cdot 4H_2O$ (0.85 g/L), $CoCl_2 \cdot 6H_2O$ (0.14 g/L), $CuCl_2 \cdot 2H_2O$ (0.085 g/L), H_3BO_3 (0.17 g/L), $ZnSO_4 \cdot 7H_2O$ (0.9 g/L), $Na_2MoO_4 \cdot 2H_2O$ (0.09 g/L).

To study the effect of medium and substrate on CO production, bacterial strains were grown in 125-mL flasks containing 10 mL of medium and incubated overnight in a rotary shaker at 37 °C. High cell density cultures were performed in a 2-L bioreactor. The dissolved oxygen was maintained at 20% of air saturation by manually increasing the air flow rate and automatically adjusting the stirrer speed. The pH was regulated at 6.8 by automatic addition of 15% (w/v) aq NH_3. Unless otherwise indicated, the temperature was maintained at 34 °C. The initial culture volume was 1 L. The initial glycerol concn was 17.5 g/L. The culture was started by the inoculation of 20 mL of a preculture on LB medium which has been incubated on a rotary shaker at 37 °C for 10 h. After consumption of the initial glycerol indicated by a sudden increase in the dissolved oxygen level, the feeding was started with an initial flow rate of 9 mL/h. The feeding soln contained: glycerol (500 g/L), $MgSO_4 \cdot 7H_2O$ (12 g/L), trace mineral soln (10 mL/L), antifoam 426 R (Prolabo) (10 mL/L).

Construction of pBC and pC5.—A translational fusion between *lacZ* and *nodB* was obtained by subcloning an *Eco*130I blunt-ended fragment from pUCNBC [13] into the *SmaI* site of pUC18. The resulting construction (pB) codes for a chimaeric protein containing the first 11 amino acids from LacZ and the complete NodB sequence without the first three amino acids (Fig. 1). To obtain pBC, the fragment *PstI-SphI* from pUCNBC was cloned symmetrically in *PstI-SphI*-digested pUB, taking advantage of the fact that the *PstI* site from the vector is very close to the *SphI* site, and is not cut when the vector is predigested with *SphI*, due to the short length of the 3′ end. To construct pC5, the *SacI-PaeI* fragment from pUCNBC, comprising *nodC* flanked by 150 bp from *nodB* and 50 bp from *nodS*, was subcloned into *SacI-Pae*I-digested pUC18.

Preparation of cell extracts.—Depending on the cell density, between 75 μL and 3 mL of culture sample was centrifuged (5 min, 12,000 *g*) in microfuge tubes. The supernatant was discarded and the pellet was resuspended in 150 μL of distilled water. After boiling for 30 min, 10 μL of concd HCl was added. Cell debris and precipitated proteins were eliminated by centrifugation (5 min, 12,000 *g*).

Acid hydrolysis of COs and colorimetric determination of N-*acetyl*-*glucosamine.*—80 μL of cell

extract or of CO sample were transferred to a microfuge tube with screw closure and O-ring seal. After addition of 40 μL of concd HCl, the tube was tightly closed and incubated for 3 h at 100 °C. The HCl was then eliminated by evaporation to dryness under an air flow at 40 °C. The products of the hydrolysis were solubilised in 80 μL of distilled water, and the GlcNAc content was colorimetrically determined after acetylation according to the method of Reissig et al. [34] using GlcNAc or purified **2** as standard.

Purification of compound **2** *from high cell density culture of strains DH1 (pUCNBC) and DH1 (pBC).*—Culture medium (1 L) was collected at the end of the fermentation and was centrifuged for 20 min at 12,000 *g*. The supernatant was discarded and the pellet was resuspended in 1 L of distilled water and autoclaved 45 min at 100 °C. After homogenisation, cooling, and an other centrifugation for 20 min at 12,000 *g*, the supernatant was mixed with an equal quantity (120 g) of powder of vegetal active charcoal (Norit) and Celite. The slurry was filtered on Whatman no. 4 paper and washed thoroughly with distilled water to remove the salts. The adsorbed oligosaccharides were then recovered by stepwise elution with 1 L each of 10% (v/v), 30% (v/v), and 40% (v/v) aq EtOH [35]. For determination of deacetylation yield after 24 h of cultivation, the procedure was scaled down for purification of 30 mL of culture medium.

The 30% (v/v) EtOH fraction was directly loaded onto a 2-cm × 10-cm column of Dowex 50 WX4 (H^+ form). After washing with distilled water (200 mL), positively charged oligosaccharides were eluted with 2% aq NH_3. Both fractions were dried by rotatory evaporation and weighed. The powder was kept at 4 °C. For structural studies compound **2** was purified by reversed phase HPLC (Lichrosorb RP18, 5–20 μm; E. Merck, Darmstadt) eluted with water. Optical rotations were measured at 20 °C on a Perkin–Elmer 241 polarimeter.

Purification of compound **1** *from high cell density culture of strain DH1 (pC5).*—Compound **1** was purified as compound **2**, except that the Dowex step was omitted and the charcoal elution was performed with 1 L of 50% (v/v) aq EtOH to reduce the elution volume and increase the purification yield.

HPLC analysis of CO production.—In order to avoid the elution of the oligosaccharides in two peaks corresponding to the α and β anomers, the products were reduced with $NaBH_4$. Aliquots from the 30% (v/v) EtOH fraction recovered from charcoal purification were concd and reduced with $NaBH_4$. After neutralisation with CH_3COOH elimination of salts by adsorption to charcoal, the sample was submitted to HPLC, using a Nucleosil Capcell 5-μm column (Interchim, Montluçon, France) and eluted with ammonium acetate (50 mM, pH 8.0) at 0.6 mL/min. The eluting products were detected using a refractometer. The ratio of **2**/**1** was calculated by integration of the corresponding peaks.

Structural analysis.—The mass spectra were obtained on a Nermag R-1010C mass spectrometer Model 200 equipped with an M-Scan Wallys-type gun. For each experiment the initial volume of matrix (glycerol) was 4 μL. The products were analysed by FAB^+MS. All metastable ions [mass-analyses ion kinetic energy (MIKE)] were obtained on a VG ZAB-SEQ, at Service Central d'Analyse, CNRS (Vernaison, France). NMR spectra were recorded on a Brucker AC300 spectrometer at 300 MHz for ^1H and 75 MHz for ^{13}C.

Penta - N, N^2, N^3, N^4, N^5 - *acetyl* - *chitopentaose* (**1**).—$[\alpha]_D^{20}$ −6° (*c* 0.5, water); ^1H NMR (300 MHz, D_2O, 30 °C): δ 5.07 (d, 0.7 H, $J_{1,2}$ 2.0 Hz, H-1α), 4.45 (m, 4 H, H-1^2, H-1^3, H-1^4, H-1^5), 3.90–3.30 (m, 30 H), 1.95, 1.92 (s, 15 H, $COCH_3$); ^{13}C NMR (D_2O): δ 175.57, 175.44 (CO), 102.42, 102.25 (C-1^2, C-1^3,C-1^4, C-1^5), 95.80 (C-1β), 91.43 (C-1α), 80.65, 80.17, 79.95, 76.90, 75.51, 74.44, 73.46, 73.07, 71.00, 70.71, 70.24, 61.55, 60.95, 56.58, 56.05, 54.62 (C-2^{1-5}, C-3^{1-5}, C-4^{1-5}, C-5^{1-5}, C-6a$^{1-5}$, C-6b$^{1-5}$), 23.11, 22.88 (CH_3). FAB^+MS: *m/z* 1034 $[M + H]^+$, 1056 $[M + Na]^+$.

Tetra-N,N^2,N^3,N^4-*acetyl-chitopentaose* (**2**).—$[\alpha]_D^{20}$ −11° (*c* 0.5, water); ^1H NMR (500 MHz, D_2O, 22 °C): δ 5.07 (d, 0.7 H, $J_{1,2}$ 2.0 Hz, H-1α), 4.57 (d, 0.3 H, $J_{1,2}$ 7.0 Hz, H-1β), 4.50–4.40 (m, 3 H, H-1^2, H-1^3, H-1^4), 4.36 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1^5), 3.85–3.20 (29 H), 2.56 (dd, 1 H, $J_{2,3}$ 8.0 Hz, H-2^5), 1.95, 1.92 (s, 12 H, $COCH_3$); ^{13}C NMR (D_2O): δ 175.55 (CO), 102.57, 102.25 (C-1^2, C-1^3, C-1^4, C-1^5), 95.80 (C-1β), 91.43 (C-1α), 80.65, 80.21, 80.06, 79.95, 79.03, 77.18, 76.46, 75.65, 75.51, 73.45, 73.08, 72.95, 71.00, 70.56, 70.24, 61.62, 61.10, 60.95, 57.64, 57.12, 56.27, 56.05, 54.63 (C-2^{1-5}, C-3^{1-5}, C-4^{1-5}, C-5^{1-5}, C-6a$^{1-5}$, C-6b$^{1-5}$), 23.11, 22.88 (CH_3). FAB^+MS: *m/z* 992 $[M + H]^+$, 1014 $[M + Na]^+$.

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