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Preparation of a novel (1→4)-β-D-glycan by *Acetobacter xylinum* — a proposed mechanism for incorporation of a *N*-acetylglucosamine residue into bacterial cellulose

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A novel polysaccharide having a *N*-acetylglucosamine (GlcNAc) residue as one of the constituents was synthesized by incubation of *Acetobacter xylinum* in a modified Schramm–Hestrin medium containing lysozyme-susceptible phosphoryl chitin (P-chitin) and D-glucose. HPLC of the culture medium showed that the P-chitin added was depolymerized to monomeric and oligomeric P-chitins during the incubation, and the P-chitins with permeable sizes were utilized as a carbon source by the bacteria. ^{13}C NMR analysis revealed that the P-chitin consists mainly of GlcNAc 6-P residues. Furthermore, monomeric GlcNAc 6-phosphate was also found to enhance the incorporation of GlcNAc residues into the polysaccharide. However, no incorporation of the GlcNAc residues was observed when *A. xylinum* was incubated in a medium containing either highly phosphorylated chitin (DS = 1.90) or its oligomers produced by acid hydrolysis. © 1997 Elsevier Science Ltd

INTRODUCTION

A gram negative bacterium, *Acetobacter xylinum*, has received a great deal of attention for the production of the essentially pure form of 'bacterial cellulose' (BC) as an extracellular pellicle (Delmer, 1983). Recently, we found that the bacteria adapted to a culture medium containing *N*-acetylglucosamine (GlcNAc) produced a novel polysaccharide analogous to cellulose containing GlcNAc, when GlcNAc (Ogawa and Tokura, 1992) or ammonium chloride (Shirai *et al.*, 1994) were added to modified Schramm–Hestrin (SH) medium (Hestrin and Schramm, 1954). The novel polysaccharide was expected to be a multi-functional polymer with both chitinous and cellulosic properties such as susceptibility against both cellulase and lysozyme (Ogawa *et al.*, 1992). It also had a higher orienting tendency and Young's modulus than those of BC (Ogawa *et al.*, 1993).

For the biosynthesis of BC, numerous investigations have been performed on the pathway of carbon metabolism of *A. xylinum*. The following four enzymatic steps are recognized as the major pathways: (i) phosphorylation of D-glucose (Glc) by glucokinase (Benziman and Rivetz, 1972); (ii) transphosphorylation of glucose 6-phosphate to glucose 1-phosphate by phosphoglucomutase (Gromet *et al.*, 1957); (iii) synthesis of uridine-5′-diphosphateglucose (UDP-Glc) by pyrophosphorylase (Valla *et al.*, 1989); and (iv) polymerization of UDP-Glc by cellulose synthase (Aloni *et al.*, 1983; Ross *et al.*, 1987). During our continuing work on the synthesis and utilization of heteropolysaccharides by *A. xylinum*, our interest has been focused on the mechanisms of how the GlcNAc residue is transferred into the (1→4)-β-D-glycan produced. We now describe several mechanistic studies on the incorporation of the GlcNAc residue into BC using phosphorylated chitins as well as GlcNAc 6-phosphate as the carbon source in the culture medium.

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MATERIALS AND METHODS

General methods

Two water-soluble P-chitins (P-chitin-M1, DS = 0.58 and P-chitin-M2, DS = 0.71) were prepared through

heterogeneous or homogeneous tosylation of chitin followed by substitution with *ortho*-phosphoric acid–urea (Shirai *et al.*, 1994). Another P-chitin (P-chitin-T1) with DS = 1.90 was prepared by direct phosphorylation with methanesulfonic acid and phosphorous pentoxide (Nishi *et al.*, 1984). GlcNAc 6-phosphate disodium salt was purchased from Sigma.

GPC analysis of the culture medium was performed by the use of a Hitachi L-6000 HPLC apparatus to estimate the molecular weight of P-chitins during incubation. The portions of culture medium were taken out time-dependently and filtered through a 0.45 μm cellulose acetate membrane (Millipore) to remove the bacteria. The filtrate was loaded on two connecting columns of Asahipak GFA-30F, and eluted with water at a rate of 0.5 ml/min at 50°C. The effluent was analyzed using a Shimadzu JC-6A refractive index detector. IR spectra of pellicles were recorded with a Horiba FT-210 Fourier-transform spectrophotometer at a resolution of 4 cm^{-1}. ^{13}C NMR spectra were recorded at 75.03 MHz with a Bruker ASX-300 spectrometer for solution in deuterium oxide at 50°C.

Acid hydrolysis of phosphoryl chitin

Phosphorylated chitin (P-chitin-T1, 1.4 g) was dissolved in 1 M HCl (180 ml) and stirred at 40°C for 24 h. The solution was concentrated and dialyzed against deionized water using a cellulose tubular membrane (Viskase Sales Corp.) which fractionated molecular weight of approximately 12 000. Its outer solution was collected and concentrated, successively dialyzed through a membrane tubing (Spectrapor, 3787-H 45), of which the cut-off molecular weight was 3500. The inner solution of the tubing was lyophilized to give a medium molecular weight fraction (P-chitin-T2, 0.08 g). The outer solution was concentrated, precipitated with methanol, and dried under reduced pressure to give a low molecular weight fraction (P-chitin-T3, 0.86 g). The molecular weights estimated by a GPC method were 4000 and 300–400 for P-chitin-T2 and -T3, respectively.

Bacterial strain and culture condition

A. xylinum ATCC 10245 was subcultured in a Schramm–Hestrin (HS) medium containing GlcNAc, and repeatedly transferred to the new culture medium every 3 days to adapt GlcNAc. Aliquots (0.5 ml) of the 3 days' culture were inoculated on the SH medium (15 ml), the sugar components of which are summarized in Tables 1–3, and incubated statically at 28°C for 7 days. The pellicles produced at the surface of the cultural medium were harvested and boiled successively in 2% (w/v) aqueous solution of SDS for 3 h and in 4% (w/v) aqueous solution of sodium hydroxide for 90 min. The pellicles were rinsed extensively with deionized water until neutral. The resulting pellicles were dried on stainless steel plates at 60°C or lyophilized. Incorporated GlcNAc residues into the pellicle were estimated by amino acid analysis, after hydrolysis in 2 M hydrochloric acid at 100°C for 12 h under reduced pressure followed by evaporation over sodium hydroxide.

RESULTS AND DISCUSSION

The ^{13}C NMR data of three kinds of water-soluble P-chitins (M1, M2, and T1) are summarized in Table 4. In the NMR spectrum of P-chitin-M1, two C-6 signals were observed at δ55.9 and 63.7 while the C-3 signal was shown to overlap with C-4 and C-5 at δ71.5–73.6, suggesting that the constituents of P-chitin-M1 are GlcNAc and GlcNAc 6-P. P-Chitin-M2 revealed a similar but simple NMR spectral pattern, showing most of the hydroxyl groups at C-6 were phosphorylated. In contrast, the C-3 and C-6 signals of P-chitin-T1 appeared at lower field (δ78.6 and 63.6, respectively) indicated that GlcNAc 3,6-P was a major constituent of the polysaccharide. Table 1 shows the amounts of incorporated GlcNAc residues in the pellicle produced in SH medium containing D-glucose and the P-chitins in the ratio of 3:1. Among these water-soluble P-chitins used, P-chitin-M2 was shown to be the most effective for the incorporation of GlcNAc residues. However, a very small amount of GlcNAc residue was incorporated when either P-chitin-M1 or -T1 were added to the SH medium. The structure of GlcNAc-containing cellulose could be elucidated by physical and chemical methods (Ogawa and Tokura, 1992). In the IR spectrum of the product, both amide-I and -II bands were observed at 1650 and 1550 cm^{-1} showing the presence of an acetamido group. The degree and the site of phosphorylation seemed to be controlling factors in GlcNAc incorporation.

The average molecular weights of the phosphoryl chitins in the culture medium were measured time-dependently by GPC analyses. As shown in Fig. 1, the molecular weight of P-chitin-M2 was slightly decreased during fermentation in SH-GlcNAc medium, and the peak corresponding to the polymeric P-chitin was broadened in SH-Glc medium. Its molecular weight distribution was 2.74 at the beginning of incubation and it changed to 4.46 after 14 days. These results indicated

Table 1. Incorporation of GlcNAc residues into pellicle produced in SH-Glc medium containing 0.5% (w/v) phosphoryl chitin derivatives

Additive	D.S.	Lysozyme susceptibility	GlcNAc (mol%)
None			0.0
P-chitin-M1	0.58	+	0.4
P-chitin-M2	0.71	+ +	2.4
P-chitin-T1	1.90	—	0.5

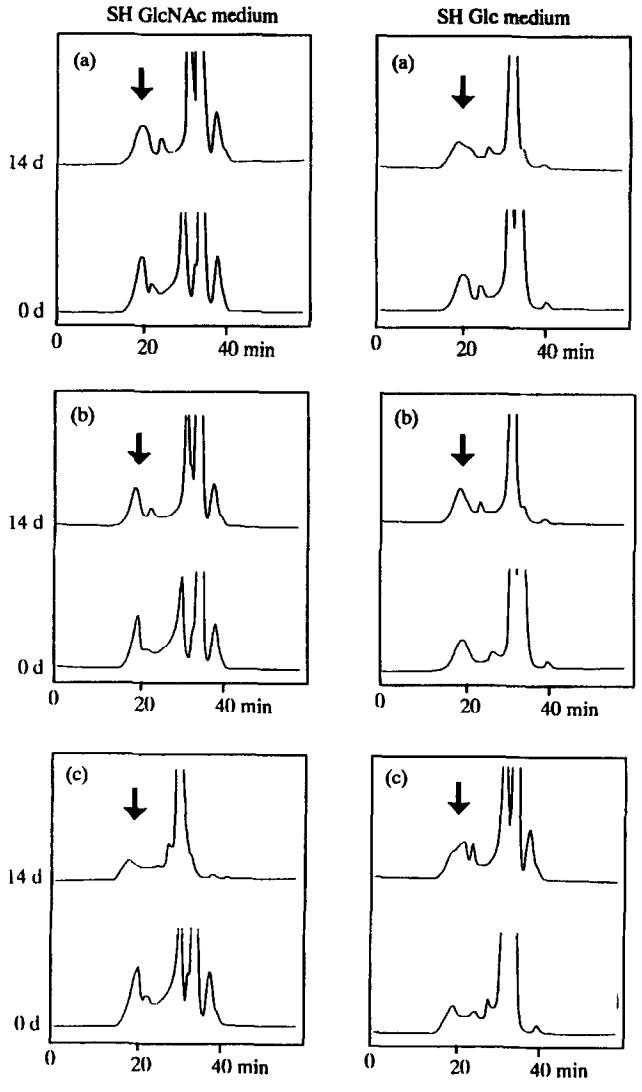


Fig. 1. GPC profiles of SH GlcNAc and SH Glc media containing 0.5 w/v% of (a) P-chitin-M1, (b) P-chitin-T1, and (c) P-chitin-M2. The arrows indicate the peak of original P-chitins eluted.

that P-chitin-M2 was degraded during the incubation. A similar tendency was observed in the case of P-chitin-T1. On the other hand, neither the molecular weight nor its distribution of P-chitin-M1 was changed in either SH-Glc or SH-GlcNAc medium. The results are comparable to the susceptibility of the P-chitins to lysozyme (Table 1). Biodegradable P-chitins seemed to be hydrolyzed by glycosidases into oligomers and/or a monomer, and consequently taken up by the bacteria as a carbon source. The oligomeric and/or monomeric P-chitins were supposed to be utilized as intermediates in the metabolism of *A. xylinum* and to be incorporated into BC.

Since low molecular weight P-chitins seemed to be precursors of the GlcNAc residues in the pellicle, we then examined the effect of addition of monomeric GlcNAc 6-P into the cultural medium. Table 2 shows the amount of incorporated GlcNAc residues after incubation in the media containing Glc/GlcNAc or Glc/GlcNAc 6-P. The incorporation was more accelerated in Glc/GlcNAc 6-P medium than in Glc/GlcNAc. Furthermore, the amount was increased with increasing ratio of GlcNAc 6-P to Glc.

In order to examine the effect of GlcNAc 3,6-P, we prepared oligomeric P-chitin from P-chitin-T1 (DS = 1.90). Thus, P-chitin-T1 was treated with 1 M hydrochloric acid at 40°C, and the hydrolysate was fractionated into two parts by dialysis. Their average molecular weights were estimated to be *ca* 4000 and 300–400 for P-chitin-T2 and -T3, respectively. The P-chitin-T3 fraction corresponded to mono- and disaccharides. These oligomeric hydrolysates were added to the SH GlcNAc and SH Glc media and similarly incubated. As shown in Table 3, however, little superiority of the oligomers for the incorporation of GlcNAc residue was observed in these experiments. Since the P-chitin-T1 is a highly substituted derivative (DS = 1.90), P-chitin-T2 and -T3 were regarded as the monomeric or oligomeric GlcNAc 3,6-P. These results indicated that GlcNAc 3,6-P or its oligomer are neither substrates nor intermediates in the metabolic pathway of the bacteria.

Hexose phosphate is regarded as a common intermediate in cellulose synthesis of *A. xylinum* (Weinhouse and Benziman, 1974; Schramm *et al.*, 1957), and it arises directly by phosphorylation of exogenous hexose (Benziman and Rivetz, 1972). The hexose phosphate would be activated as UDP-hexose in the metabolic pathway of the bacteria. Our present experiments showed that biodegradable phosphoryl chitin added to the culture medium was depolymerized to a monomer and oligomers during incubation. Furthermore, GlcNAc 6-P was found to

Table 2. Incorporation of GlcNAc residues into pellicle produced in SH Glc medium containing Glc or GlcNAc 6-phosphate

Sugar components in medium (wt)		GlcNAc in pellicle (mol%)
Glc:GlcNAc	7:3	0.3
	3:7	1.2
Glc:GlcNAc 6-P	7:3	1.3
	3:7	3.4

Table 3. Incorporation of GlcNAc residues into pellicle produced in medium containing 0.5 w/v% of acid hydrolysates of P-chitin-T1 (DS = 1.90)

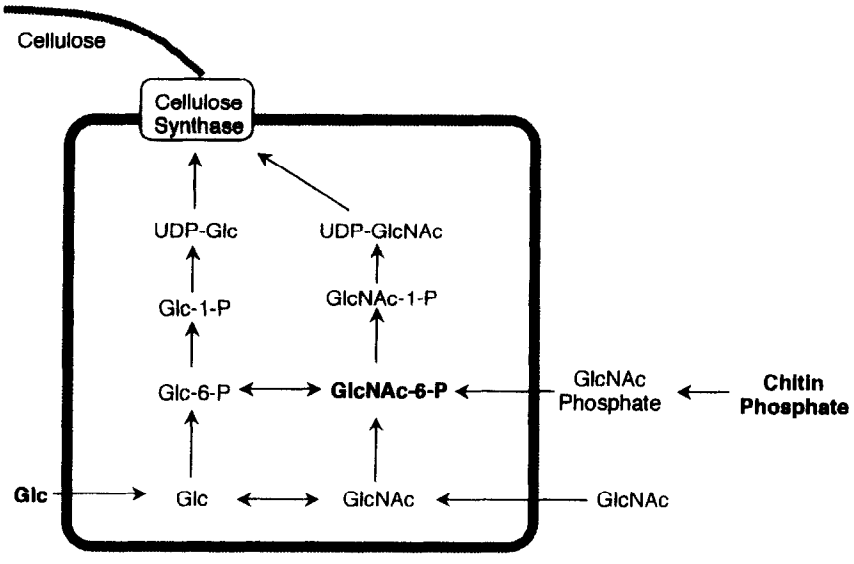
Culture medium	Additive[a]	GlcNAc (mol%)
SH GlcNAc	none	0.8
	P-chitin-T2	0.2
	P-chitin-T3	0.2
SH Glc	none	0.0
	P-chitin-T3	0.7

[a] Molecular weights of P-chitin-T2 and P-chitin-T3 estimated by GPC were 4000 and 300–400, respectively.

Table 4. Chemical shifts of ^{13}C NMR spectra of phosphorylated chitins

P-chitins	C-1	C-2	C-3	C-3′	C-4	C-5	C-6	C-6′	CH_3	C=O
T1	98.2	52.9	70.5	—	73.6	71.5	59.9	63.7	21.8	173.0
T2	100.2	54.8	70.5	—	73.3	71.6	59.8	62.6	21.9	173.0
M1	101.1	54.7	—	78.6	72.8	71.8	63.6	—	22.1	174.5

[a]^{13}C NMR spectra were recorded at 75.03 MHz in deuterium oxide at 50°C.



Scheme 1. The proposed flow of GlcNAc phosphate in the metabolic pathway of *Acetobacter xylinum*.

accelerate the incorporation of the GlcNAc residues. These facts suggest that GlcNAc 6-P plays an important role in the metabolic pathway. The flow of GlcNAc phosphate was proposed as illustrated in Scheme 1. Thus, phosphoryl chitin is initially degraded to a monomer, GlcNAc 6-P, which may be isomerized to GlcNAc 1-P. Finally, it led to UDP-GlcNAc being incorporated into the polysaccharide chain similarly to the metabolic pathway of Glc.

CONCLUSION

Water-soluble and lysozyme-susceptible P-chitin with a degree of substitution of 0.71 was found to be depolymerized in a culture medium of *Acetobacter xylinum* and then to be incorporated into its metabolic pathway. GlcNAc 6-P was also a good substrate for biosynthesis of novel BC containing GlcNAc residues. Work is in progress along this line; the objective is to prepare new polysaccharides with a variety of physical and biological properties.

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