The directionality of chitin biosynthesis: a revisit

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The molecular directionality of chitin biosynthesis was investigated by transmission electron microscopy (TEM) using electron crystallography methods applied to reducing-end-labelled β-chitin microcrystals from vestimentiferan *Lamellibrachia satsuma* tubes and nascent β-chitin microfibrils from the diatom *Thalassiosira weissflogii*. The data allowed confirmation that the microfibrils were extruded with their reducing end away from the biosynthetic loci, an orientation consistent only with elongation through polymerization at the non-reducing end of the growing chains. Such a chain-extension mechanism, which has also been demonstrated for cellulose and hyaluronan, appears to be general for glycosyltransferases that belong to the GT2 (glycosyl transferase 2) family. The data also allowed confirmation that in β-chitin the chains are crystallized in a 'parallel-up' mode, in contrast with hypotheses proposed in previous reports.

Key words: chain elongation, β-chitin, chitin biosynthesis, electron crystallography, glycosyl transferase family 2, parallel packing.

INTRODUCTION

Chitin and cellulose are abundant $(1 \rightarrow 4)$-β-linked linear homopolysaccharides of actual and potential industrial applications, whose biosynthesis and biodegradation continue to be intensively investigated. Whereas much is understood about the enzymic hydrolysis of cellulose and chitin, the mechanism of biogenesis of these polysaccharides remains mysterious, despite numerous efforts [1–4].

In our laboratory group, we became interested in devising a method to prove unambiguously whether native cellulose was being synthesized from its reducing or non-reducing end [5]. Our method was based on the extraction of individual whisker-like cellulose microcrystals from the highly crystalline *Cladophora* cell walls. In our hands, these crystals were amenable to an electron diffraction study, using a very small probe for the recording of set electron diffraction diagrams on sequentially tilted crystals that had been either stained at their reducing end [6] or eroded by Cel6A, a specific exo-type enzyme [7,8] (see also http://afmb.cnrs-mrs.fr/CAZY/). This technique allowed us to show that native cellulose was of the 'parallel-up' type configuration. The definitions of 'parallel up' and 'parallel down' are according to French and Howley [9], where the unit cell parameters must be defined in the standard convention as in Figure 1, with the ***a*** parameter directed toward the viewer, ***b*** and ***c*** in the plane of the paper, and γ is obtuse. The structure is said to be 'parallel up' when the z co-ordinate of O5 is greater than that of C5. It is 'parallel down' in the other case. This designation is in full agreement with the established crystal structure [10,11] and modelling of cellulose [12]. Thus we could show that the reducing ends of the cellulose chains in the microcrystals were directed toward the tip of the ***c*** parameter of the unit cell (Figure 1). Adapting this electron-diffraction identification method to growing bacterial cellulose microfibrils still attached to their synthesizing loci, we could demonstrate without ambiguity that, during cellulose biosynthesis, the monomers were added by insertion at the non-reducing end of the microfibrils [5]. This observation confirmed related glycosyl transferase crystallography findings, where the directionality of insertion of nucleotide sugars within catalytic sites of enzymes similar to those of cellulose synthase could be evidenced [13–15].

β-Chitin bears substantial similarity to native cellulose. It consists also of slender crystalline microfibrils [16,17] where the chitin chains are packed in a parallel fashion, with all their reducing ends pointed towards the same direction in a given microfibril [18,19]. Some specimens of β-chitin are of extreme crystallinity, such as the microfibrils from the tubes of vestimentiferan *Lamellibrachia satsuma* or the spines from the centric diatom *Thalassiosira weissflogii*. As in the case of native cellulose, the β-chitin microfibrils can be hydrolysed with HCl to yield well-defined micron-sized chitin microcrystals whose width is the same as that of the parent microfibrils [17]. As these microfibrils or microcrystals yield well-resolved electron-diffraction patterns, they should be easily amenable to a technique similar to that of the cellulose reducing-end labelling and electron-diffraction identification. As for cellulose, one should therefore be in a position to probe the sense of extension of the chitin molecules during their biosynthesis.

Despite the apparent simplicity of this problem, conflicting observations have been made when we tried to match the reducing-end direction of the chitin chains with the directionality of the ***c*** parameter of the β-chitin unit cell. At first, it was not possible to stain the reducing-end tip of the β-chitin microcrystals, and we had to rely on the pointed-tip geometry of the erosion of the crystals when they were subjected to the digestion of the chitinase A1 [GH18 (glycoside hydrolase family 18)] [8] (see also http://afmb.cnrs-mrs.fr/CAZY/) from *Bacillus circulans* [19]. From the results of the digestion by this enzyme of soluble *p*-nitrophenyl chitodextrins, we concluded erroneously that chitinase A1 was degrading β-chitin microcrystals from their non-reducing ends. Indeed, our initial deduction was shown to be wrong when adequate reducing-end staining of the microcrystals was subsequently developed [19]: the resulting images proved that chitinase A1 was in fact operating at the reducing ends of β-chitin crystalline substrates. The determination of the directionality of the ***c*** vector of β-chitin from electron-diffraction and tilting

Abbreviations used: GT2, glycosyl transferase 2; TEM, transmission electron microscopy.

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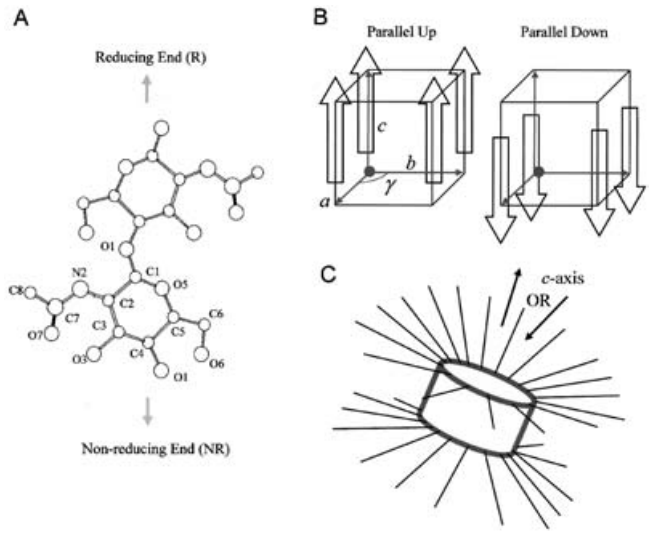


Figure 1 Key structural features in the β-chitin system discussed in the present study

(**A**) Chitobiosyl residue with identification of the reducing and non-reducing ends. (**B**) Two modes of packing of chitin molecules in the β-chitin unit cell. The parallel-up (left) model was proposed by Gardner and Blackwell [20], whereas a parallel-down structure was suggested earlier by Sugiyama et al. [18]. (**C**) A schematic drawing of a diatom cell spinning chitin. The chitin microfibrils (or spines) are extruded from protruding pores located on the silicate body. This system is well-adapted to identify the directionality of the biosynthesis of chitin.

experiments proved also to be difficult, and our first deduction was that β-chitin was of the parallel-down structure (the non-reducing end of chitin molecules was toward the tip of the ***c*** parameter of the unit cell). This deduction contradicted the precise crystal structure determination made by the Blackwell group [20], which has shown that a parallel-up structure was occurring in β-chitin. As for cellulose, the implication of the parallel up or parallel down structure of β-chitin is important to determine the molecular directionality of chitin biosynthesis.

The present study was devised to clarify the directionality of the biosynthesis direction in β-chitin. First, the direction of the ***c*** parameter of the unit cell was confirmed using microcrystals from β-chitin of *L. satsuma* labelled at their reducing-end tips. Secondly, the direction of the ***c*** parameter was identified in β-chitin microfibrils synthesized from *T. weissflogii*. As seen in the present paper, these experiments allowed us to prove without ambiguity that β-chitin was biosynthesized by insertion of monomers at the non-reducing ends of the growing chitin chains.

MATERIALS AND METHODS

Preparation of β-chitin sample

The degradation experiment by chitinase A1 was achieved with β-chitin microfibrils extracted from tubes of vestimentiferan, *L. satsuma*, which were collected in Kagoshima Bay, Kyushu, Japan [21]. A formaldehyde-fixed tube was deproteinized by two cycles of alkali and bleaching treatments. The samples were soaked in 5 % (w/v) KOH at room temperature (25 °C) overnight, and then in 0.3 % (w/v) $NaClO_2$ in sodium acetate buffer, pH 4.9, at 70 °C for 4 h. Purified tubes were hydrolysed by boiling in 2.5 M HCl with continuous strong stirring for 4 h. The resultant suspension of chitin microcrystals was neutralized by several centrifugal washes (14000 ***g*** for 5 min) with distilled water and finally dialysed overnight under tap water, and for 1 day in distilled water. The microcrystals were then degraded by chitinase A1, the major chitinase in *Bacillus circulans* [22,23], and subsequently their reducing ends were visualized as in the previous study [19].

A centric diatom, *T. weissflogii*, was used to clarify the elongation directionality of the chitin molecules. The diatom was cultured in artificial seawater (Marine Art; Senju Seiyaku Co., Japan) enriched with f/2 medium ([24]; http://ccmp.bigelow.org/CI/f2_family.htm) for 1 week, following a day and night cycle of 16 h and 8 h at 28 °C. At the end of the culture period, the cells were fixed with 100 mM sodium cacodylate buffer, pH 7.0, for 2 h at room temperature. The fixed samples were immediately washed by dialysis against distilled water, without any chemical purification procedures. A specimen containing numerous chitin microfibrils was deposited on a carbon-coated copper grid just before transmission electron microscopy (TEM) observation.

TEM observation

All the micrographs and diffraction diagrams were taken with a JEOL-2000EXII (Jeol, Tokyo, Japan) operated at 100 kV and recorded on Mitsubishi MEM microscopic film (Mitsubishi Paper Mills, Tokyo, Japan). Diffraction contrast imaging in bright-field mode was used to visualize the sample without further contrast enhancement. The images were taken at 2500–6000× under low-dose exposure with the use of a Minimum Dose System (MDS; Jeol).

Microdiffraction experiment with tilting

All the diffraction diagrams were obtained in the microdiffraction mode. For this, a small condenser aperture of 20 μm was inserted in the second condenser lens and the first condenser lens was fully overfocused to achieve an electron probe of approx. 100 nm. The samples were observed at 2500× under extremely-low-dose conditions with the help of an image intensifier (Fiber Optics Coupled TV; Gatan, Pleasanton, CA, U.S.A.). When a microcrystal was found, it was rotated to align its long axis perpendicular to the microscope tilt axis by using the rotation-tilt holder (SRH holder; Jeol). Tilt angles ranging from $-20°$ to $+20°$ were set to record meaningful diffraction patterns (see Figures 2 and 4). Sets of tilted diffraction patterns were recorded from the nearest portion of the single microcrystal.

RESULTS

Re-examination of the structure of β-chitin

As shown in the previous report [19] and in Figure 2(A), the digestion of chitin from *L. satsuma* by chitinase A1 induces a tapering at the reducing-end tip of the chitin microfibrils. The strategy to identify the direction of the ***c*** parameter of the unit cell is basically identical with that developed in previous reports [5,18] where a tilting-diffraction experiment was employed for microfibrils giving single-crystal-like net patterns. In the present study, the tilt axis was not along the fibre axis of microfibril as previously suggested, but was perpendicular to the fibre as shown in Figure 2. An advantage of this strategy is that a single tilt is adequate to define the direction of the ***c***-axis. Therefore tilting the single microfibril in both orthogonal directions will serve as a double assignment.

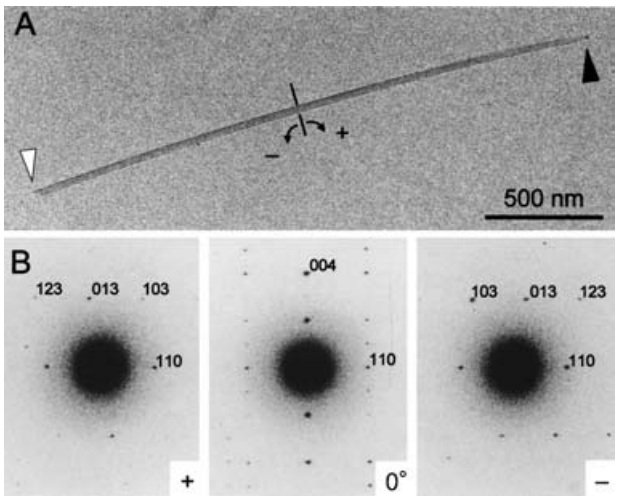


Figure 2 β-Chitin microfibril that was hydrolysed by chitinase A1 enzyme (A), together with a series of tilting-microdiffraction diagrams taken from this microfibril (B)

(**A**) The reducing ends in the microfibril are visualized by colloidal gold (arrowhead) specifically at the tapered tip of microfibril. (**B**) Electron diffraction diagrams taken from the tapered microfibril in (**A**), each of them tilted by $\boldsymbol{ca} + 17°$, 0° or $-17°$ around the axis perpendicular to the fibre: the plus tilting allows the tapered tip go down from the observer while the minus brings it up to the observer. The diffractions obtained are indexed as shown. Note that the two cases of $+17°$ and $-17°$ of tilting are clearly distinguishable from each other in the diffraction pattern.

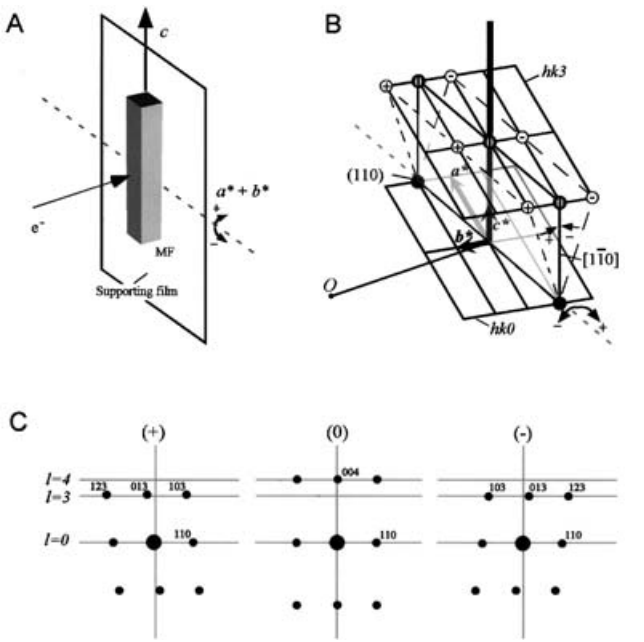


Figure 3 Theoretical background of the tilting-diffraction experiment shown in Figure 2(B)

(**A**) Schematic diagram of *L. satsuma* β-chitin microfibril. The dominant equatorial diffraction was 110 in this case, meaning that the microfibril was tilted around the $\boldsymbol{a}^* + \boldsymbol{b}^*$ vector for the series of electron diffraction. MF, microfibril; e^-, incident electron beam. (**B**) and (**C**) Corresponding reciprocal net of a crystal in (**A**) and the expected diffraction patterns. By tilting $\pm 17°$, one can bring (103), (013) and (123) to be diffracted; 110 appears on the equator in each case. Circles with +, 0 and − in (**B**) correspond to the diffractions that appear in Figure 2(C) by the plus, 0° and minus tiltings respectively. These diffraction spots on the third layer line are diagnostic of the direction of $\boldsymbol{c}$-axis as shown in (**C**): the diffraction pattern at the left indicates the $\boldsymbol{c}$-axis going away from the observer, and the pattern at the right has the $\boldsymbol{c}$-axis coming up to the observer.

Typical sets of tilting-electron-diffraction experiments with *L. satsuma* β-chitin microfibril frequently gave a 110 reflection on the equator (Figure 2B). Thus the tilting axis was around the $\boldsymbol{a}^* + \boldsymbol{b}^*$ vector, the corresponding reciprocal net shown in Figure 3(B). Tilting by 17° introduces diffractions from three crystallographic planes in addition to (110), i.e. (013), (103) and (123). Two distinguishable diffraction patterns which are mirror images of each other are obtained depending on the rotational direction, as shown in Figure 3(B). When tilting brings the $\boldsymbol{c}$ parameter away from the observer ('plus tilting'), the diffraction spot of 013 appears in the top-left and the bottom-right of the diagram. In contrast, the spot will appear on the left-bottom and the right-top with 'minus tilting'. The directionality of the $\boldsymbol{c}$ parameter can thus be experimentally determined. More than 20 sets of tilting-diffraction experiments, as shown in Figure 2(B), clearly showed that the $\boldsymbol{c}$ parameter goes toward the tip tapered by chitinase A1 degradation, i.e. the reducing-end tip. Therefore the packing of the chitin chains in the β-chitin unit cell are established as parallel-up, which is supported by computational modelling [25] and crystallographic analysis [20].

Molecular directionality of β-chitin biosynthesis

Diatom spines, which are made up of highly crystalline β-chitin [16,26,27], are known to have a peculiar microfibrillar feature. Herth and Zugenmeier [16] have pointed out that one can distinguish distal and proximal ends, because the former are much thinner and long-tapered than the latter (Figures 4A and 4B). Thus a microscopic observation allows us to identify which tip points towards the biosynthetic site, even in spines isolated from the parent body.

A standard tilting-diffraction experiment was performed for such spines that exclusively gave 1$\bar{1}$0 diffraction on the equator (Figure 4C). Then the crystal was tilted around $\boldsymbol{a}^* - \boldsymbol{b}^*$ vector in this case (see Figures 5A and 5B). Tilting by 12° introduces diffractions from three crystallographic planes in addition to (1$\bar{1}$0), i.e. (015), (105) and (1$\bar{2}$5). As the experiment on *L. satsuma* microfibrils, diffraction patterns with plus and minus tilting are mirror images of each other (Figure 5C). When tilting brings the $\boldsymbol{c}$-axis to the side away from the observer (plus tilting), the diffraction spot of 015 appears in the left-bottom and the top-right of the diagram, whereas minus tilting brings the spot on the top-left and the bottom-right. We performed the experiment on 20 spines. All diffraction patterns were without any exception as shown in Figure 4(C), showing that the $\boldsymbol{c}$-axis points toward the distal end of the spine. Thus, as is the case with cellulose I microfibril [5], the distal end of spine is unambiguously assigned as the reducing end of the parallel β-chitin microfibril.

DISCUSSION

In the present study, we have been able to confirm that chitinase A1 could digest highly crystalline β-chitin, starting from their reducing tips. Thus we are in a position to correct our earlier hypothesis where a non-reducing-end attack was suggested from a study on the biodegradation of soluble chito-oligosaccharides. [18]. As proposed in our earlier paper [18], the present work proves also without any ambiguity that in β-chitin the molecules are synthesized by the insertion of precursors at the non-reducing

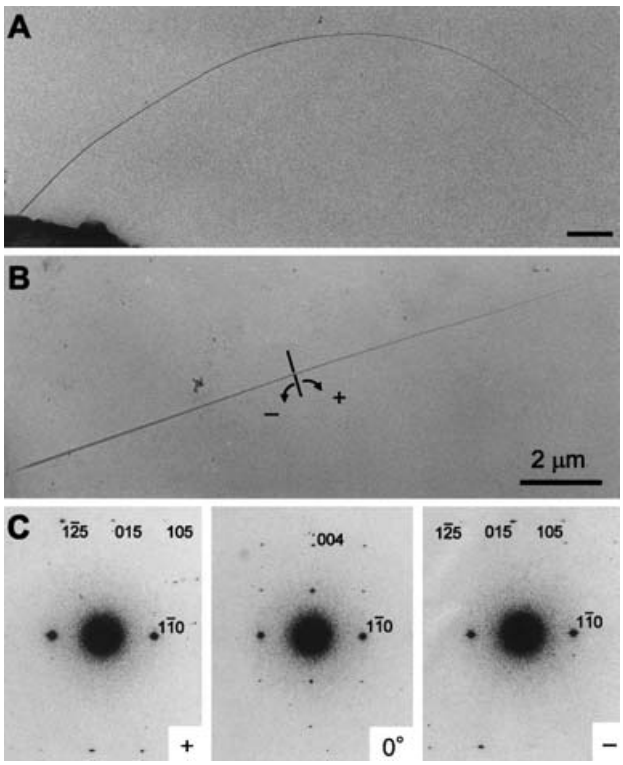


Figure 4 β-Chitin diatom spine of *T. weissflogii* (A) and (B), together with a series of tilting-diffraction diagrams taken from this spine (C)

(**A**) A spine is extruded from silica body of *T. weissflogii*. (**B**) An isolated spine clearly showing distal (right) and proximal ends (left). (**C**) Electron-microdiffraction diagrams taken from the single spine that is tilted by + 12°, 0° and − 12° around the axis perpendicular to the fibre, as indicated in (**B**): the plus tilting allows the distal end to go away from the observer, and the minus brings it up to the observer. The diffractions obtained are indexed as shown. As in Figure 2(C), the obtained diffraction patterns are distinguishable from each other.

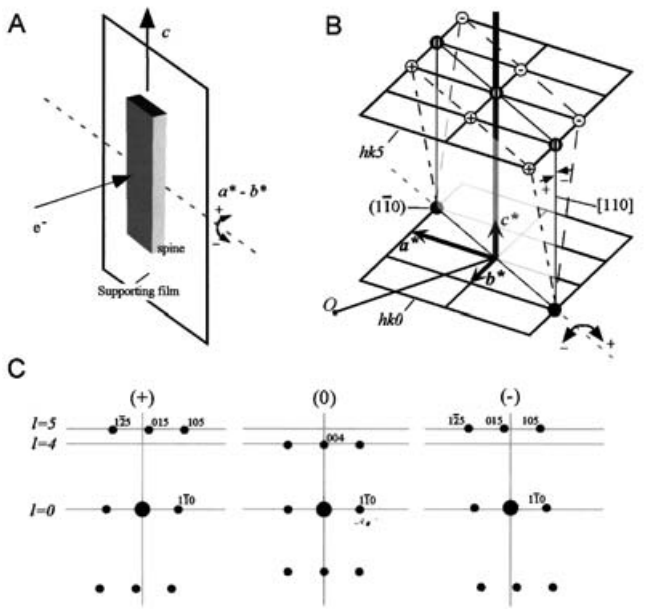


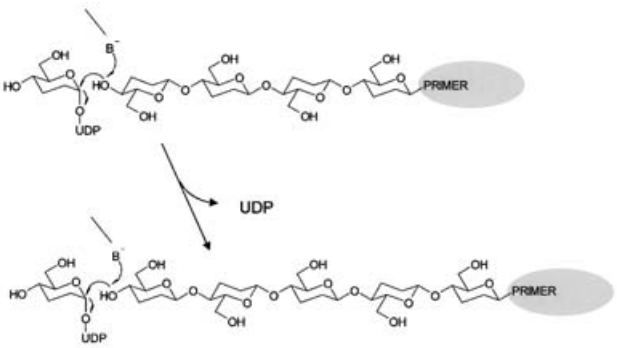
Figure 5 Theoretical background of tilting-diffraction experiment shown in Figure 4(B)

(**A**) Schematic diagram of *T. weissflogii* spine of β-chitin. The dominant equatorial diffraction was 1$\bar{1}$0 in this case, meaning that the spine was tilted around $\boldsymbol{a}^* - \boldsymbol{b}^*$ vector in this experiment. (**B**) and (**C**) Corresponding reciprocal net of a crystal in (**A**) and the expected diffraction patterns. By tilting $\pm$ 12°, one can bring (105), (015) and (1$\bar{2}$5) on to the Ewald sphere (to be diffracted); 1$\bar{1}$0 appears on the equator in each case. Circles +, 0 and − in (**B**) correspond to the diffractions that appeared in Figure 4(C) by the plus, 0° and minus tilting respectively. These diffraction spots on the fifth layer line are diagnostic of the direction of the $\boldsymbol{c}$-axis as shown in (**C**): the diffraction pattern at the left indicates that the $\boldsymbol{c}$-axis is going away from the observer, and the pattern at the right that the $\boldsymbol{c}$-axis is coming up towards the observer.

end of the growing microfibrils. Finally, we confirm that β-chitin microfibrils consist of crystalline domains where the polymer chains are assembled in the parallel-up system.

In β-chitin, the growth from the non-reducing end is consistent with related observations made on the biosynthesis of other polysaccharides, namely those of cellulose [5,28] and hyaluronan [29], for which a non-reducing-end type of biosynthesis has also been reported. Quite remarkably, both cellulose synthases, as well as hyaluronan synthases, belong to the GT2 family of the glycosyl transferases, where chitin synthase is also found [30]. In a given glycosyl transferase family, it is generally accepted that the stereochemistry of the enzymic mechanism and in particular that of the sugar insertion is maintained for the whole family. Thus, if one of several three-dimensional structures can be solved for the proteins of a given family, their features and catalytic mechanism can be applied to other members of the same family. The three-dimensional structures of several glycosyl transferases have been solved [13–15]. Among these structures, that of SpsA from *Bacillus subtilis* is pertinent to this work, as this enzyme belongs to the GT2 family [15]: this protein is involved in the production of the mature spore coat during the spore response of the bacterium. The structure of its complex crystal with UDP and glycerol has revealed that a monosaccharide hooked to UDP is added to the non-reducing end of the substrate molecule. In addition, the nucleotide-phosphate binding and catalytic residues in inverting-type glycosyl transferases from other families including GT2 were found to match each other spatially [13], implying a conserved binding and catalytic machinery in these families. Among them, the atomic structure of GlcAT-I (1,3-glucuronyltransferase I) clearly showed that the acceptor molecule of oligosaccharide with its non-reducing hydroxy group faced the distal phosphate of UDP [31], supporting the idea that the donor sugar is transferred to the non-reducing end of the acceptor. The accumulation of these features indicates that the chain elongation at non-reducing end seems to be a common mechanism of polysaccharide biosynthesis by glycosyl transferases from the GT2 family.

When looking through the literature, other hypotheses for the biosynthesis of polysaccharides have been proposed. Some authors have proposed a reducing-end monomer insertion for cellulose [32] and hyaluronan [33] biosynthesis. Despite their pertinence, these studies have used crude synthases isolated in microsomes extracted from cultured cells of model organism. In addition, theses studies rely on indirect analysis of the resulting product, namely the radioactivity counting of the degraded *in vitro* product. In contrast, more recent studies on the biosynthesis of NodC [34] and PmHas [29] have used purified recombinant enzymes and directly characterized the product to determine the directionality of synthesis. The chitin synthase of Nod-factor is involved in the formation of root nodules on a host plant in symbiosis with soil bacteria, whereas hyaluronan synthase operates in Gram-negative bacteria. Both synthases are relevant to cellulose and chitin synthases in that they catalyse the glycosyl transfer of UDP-GlcNAc and that, in both of them, the monomer is added



Scheme 1 Elongation of the polysaccharide chain with a primer molecule attached to the reducing end

The synthase adds single monosaccharides in a sequential fashion to the non-reducing ends of the growing nascent polysaccharide chain. The simultaneous formation of the disaccharide repeat unit as postulated previously [5,37] is not supported by current knowledge. The reducing ends may carry the primer-type molecule, such as UDP, lipid-phosphate, sterol or other proteins.

at the non-reducing ends of the growing chains. Therefore, the contradictory studies [32,33] may include other events concerning the polysaccharide biosynthesis, such as the formation and/or conversion of intermediates, e.g. lipid phosphate.

The molecular directionality in the diatomaceous β-chitin spine was determined in this study by electron crystallography. Although the direct labelling of the reducing end on the spine would have been much simpler for this purpose, we could not put it to work. Nevertheless, the labelling was found at both ends of spine when they were oxidized by periodic acid before labelling: this pre-treatment introduces aldehyde groups at both reducing and non-reducing ends [19]. We therefore suspect that the reducing end of chitin was covered by a non-chitinous substance, as schematically shown in Scheme 1. Such foreign material appears to be removed by periodic acid oxidation, leading to the exposure of the underlying aldehyde groups. An activated group or carrier molecule resulting from the initial glycosyl transfers is a likely candidate for such non-chitinous material. Similarly, with the biosynthesis of glycogen [35] and cellulose [36], which use respectively glycogenin and sitosterol for primers, we could conceive that the biosynthesis of chitin also requires a protein or a lipid aglycone as a synthesis primer. A biochemical analysis of the non-chitinous material located at the reducing tip of the β-chitin spines could therefore give us some hints about the identification of the chitin biosynthesis primer.

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