Tunicamycin Prevents Cellulose Microfibril Formation in *Oocystis solitaria*¹

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**ABSTRACT**

The effect of tunicamycin (TM) on the development of the cell wall in *Oocystis solitaria* has been investigated. It was found that 10 micromolar TM completely stops the assembly of new microfibrils as observed at the ultrastructural level. During cell wall formation, freeze fracture replicas of the E-face of the plasma membrane reveal two major substructures: the terminal complexes (TC), paired and unpaired, and the microfibril imprints extending from unpaired TCs. In cells treated for 3 hours or longer with TM, the TCs are no longer visible, whereas microfibril imprints are still present. Because of the reported highly selective mode of action of TM, our results implicate a role for lipid-intermediates in cellulose synthesis in *O. solitaria*. It is assumed that TM prevents the formation of a glycoprotein which probably is a fundamental part of the TCs and may act as a primer for the assembly of the microfibrils.

It is generally accepted that cellulose is assembled at the plasma membrane surface and that UDP-glucose serves as the basic sugar donor (2, 3, 12). Unknown is how the glucose units reach the site of polymerization. Most intriguing is the idea that lipid-linked glucose moieties or complex lipid-linked oligosaccharides may play a role as intermediaries in cellulose synthesis as was first postulated by Kahn and Colvin (9) for bacterial cellulose. The lipids in question are of the polysoprenyl type and are named dolichol in eucaryotic systems (4).

TM completely blocks the UDP-GlcNAc: dolicholphosphate GlcNAc-1-phosphatetransferase, the first enzyme of the dolichol pathway (11, 23) and thereby prevents the N-glycosylation of polypeptides at asparagine residues. TM, at a concentration about 100× higher than necessary to block the transferase, also inhibits the transfer of glucose and galactose from the corresponding nucleosides diphosphate sugar to dolicholphosphate (23). Such a reaction involving glucose transfer has been proposed to occur in cellulose synthesis in *Acetobacter vinelandii* (2) and in *Prototheca zopfii* (8).

In recent years many ultrastructural and physiological aspects of cellulose microfibril formation in the green algae *Oocystis solitaria* have been elucidated (16, 20).

Cortical microtubules control the regular change in the direction of extracellular microfibril deposition (14, 17). This coincides with a 90° change in the directional insertion of intramembranous particles, the so-called TCs, into the plasma membrane (1, 16). On the basis of experiments carried out with inhibitors of microfibril assembly (19), the putative cellulose synthesizing function of the TCs has been confirmed and elaborated on (15, 18).

In this report the effect in vivo of TM on cellulose microfibril deposition and on the appearance of TCs at the E-face of the plasma membrane in *O. solitaria* is described.

**MATERIALS AND METHODS**

The algae were grown, fixed, embedded, sectioned, and stained for TEM examination as described previously (17). The freeze fracture investigations were conducted as described by Robinson and Quader (19).

The algae were treated with TM (Sigma, Munich) under normal growth conditions subsequent to a 6-h colchicine treatment (10⁻³ m; Sigma, Munich). TM was dissolved in DMSO resulting in a maximal DMSO concentration of 1% in the culture medium. For recovery experiments, the treated cultures were extensively washed with culture medium by centrifugation.

The cellulose measurements were carried out as described by Quader *et al.* (18). Protein synthesis was studied by the capability of young autospores to incorporate [³H]leucine into TCA-precipitable proteins in the absence or presence of TM (10 μM). At the appropriate time intervals, aliquots (500 μl) of cells incubated with [³H]leucine (3.7 × 10⁴ Bq/ml) were taken, mixed with 10 volumes of medium, and collected on Whatman glass fiber filters which were extensively rinsed with medium. The obtained values represent the uptake of [³H]leucine.

TCA-precipitable proteins were determined by adding 1 volume of 10% TCA to aliquots of disintegrated cell suspensions. The precipitates were collected on glass fiber filters which were counted in a Philips scintillation counter using Supersolve counting solution (Zinser, Frankfurt).

**RESULTS**

Inhibition of Microfibril Deposition. *Oocystis solitaria* cultures do not show a fully synchronous growth (18). Therefore, to mark the developmental state of wall development in young autospores, TM was applied after a 6-h colchicine treatment (10 mm; 17). Autospores normally regain the capability to develop a regularly changing pattern of microfibrillar layers after colchicine has been washed out, thereby producing a sandwich-like wall (Fig. 1a; 17). As displayed in Figure 1a, within a 14-h postcolchicine recovery, about 12 to 15 microfibril layers are deposited. With 10 μM TM (the lowest fully effective concentration) in the recovery medium, no microfibrils are laid down after the colchicine period (Fig. 1b). Concentrations below 2 μM were ineffective, whereas the inhibitory effect of concentrations between 2 and 10 μM was clearly reduced. Whereas TM apparently interferes with the regular synthesis of cellulose, microfibrules
FIG. 1. Cell wall of O. solitaria autospores treated for 6 h with 10 mM colchicine followed by a 15-h recovery period during which different treatments were made. Reassembled cortical microtubules are indicated by arrows. Bar = 0.5 μm. (a), Normal recovery after colchicine treatment. A sandwich-like wall arrangement is produced (x 56,500). (b), Recovery in the presence of TM. No microfibrils are laid down after the colchicine treatment (x 46,800). (c), Recovery (7 h) in the presence of TM, then given an extra 8 hr in the absence of TM. Arrow head, end of the colchicine as well as the TM treatment (x 46,800).

Table I. Cellulose Synthesis in the Presence of TM
The amount of cellulose is given as μg/10⁷ cells. Each number represents the mean of triplicate determinations.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Cellulose Production in the Presence of</th>
<th>None</th>
<th>TM (10⁻⁵ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>11.83</td>
<td>10.43</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>16.67</td>
<td>11.47</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>23.77</td>
<td>12.43</td>
<td></td>
</tr>
</tbody>
</table>

Table II. [³H]Leucine Incorporation into Proteins in the Presence of TM
For comparison, the results obtained with the translation inhibitor cycloheximide are also shown. The values of triplicate determinations are presented as per cent of the amount of [³H]leucine taken up by the cells at the indicated time intervals.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[³H]Leucine Incorporated after</th>
<th>0 min</th>
<th>90 min</th>
<th>180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>&lt;1</td>
<td>8.9</td>
<td>14.3</td>
<td></td>
</tr>
<tr>
<td>TM (10⁻⁵ M)</td>
<td></td>
<td>9.3</td>
<td>11.2</td>
<td></td>
</tr>
<tr>
<td>Cycloheximide (20 μg/ml)</td>
<td></td>
<td>4.3</td>
<td>3.2</td>
<td></td>
</tr>
</tbody>
</table>
FIG. 2. Freeze-fracture replicas (E-face) of the *O. solitaria* plasma membrane in the presence or absence of 10 \( \mu M \) TM. (a), E-fracture face of the plasma membrane of an untreated cell. Unpaired TCs with attached microfibrils are indicated by a single arrow; paired TCs by a double arrow \((\times 29,000)\). Bar = 1.0 \( \mu m \). (b), Two h TM treatment. The dense packing of the microfibril imprints indicates that the microfibril layer is almost complete. Arrows indicate the TCs as described for 'a' \((\times 35,000)\). Bar = 0.5 \( \mu m \). (c), Typical E-fracture face of the plasma membrane of a cell treated for 3 h with TM. Only short and long microfibril imprints without TCs are visible (arrows) \((\times 21,000)\) Bar = 1 \( \mu m \).

Nevertheless reappear subjacent to the plasma membrane after colchicine has been removed (Fig. 1, a–c).

Results obtained by chemically determining cellulose deposition during cell wall formation are in full agreement with those obtained through ultrastructural observations (Table I).

Recovery from TM Treatment. The TM effect is fully reversible. Removing TM from the postcolchicine culture medium after 7 h results in the deposition of five to seven new microfibril layers within the following 7 h. These microfibrils are laid down in the normal alternating pattern (Fig. 1c).

Protein Synthesis in the Presence of TM. The effect of TM on the incorporation of \([^{3}H]leucine\) into the TCA-precipitable protein fraction was studied to elucidate if the observed effect of TM is due to a nonspecific inhibition of protein synthesis. About 10% of the \([^{3}H]leucine\) taken up by the cells, normally between 5% and 8% of the applied amount of \([^{3}H]leucine\), is incorporated...
into the protein. Whereas cycloheximide (20 μg/ml) obviously prevents the incorporation of the labeled amino acid, TM (10 μM) apparently affects [3H]leucine incorporation not at all or only slightly (Table II). After a 3-h incubation period, the values in the presence of TM were between 75% and 90% of the untreated cells.

Effect of TM on Structural Features of the Plasma Membrane.
Two distinct structural features can be observed on freeze fracture replicas in the plane of the plasma membrane E-face: imprints of extracellularly deposited microfibrils and TCs (Fig. 2a; 16). Depending on the duration of the TM treatment, two observations were made. After 1 to 2 h, paired and unpaired TCs, the latter connected to microfibril imprints, are still to be seen, but only very faintly (Fig. 2b). With few exceptions, treatments longer than 3 h prevent the occurrence of both types of TCs, whereas the microfibril imprints without TCs (see Fig. 2c), apparently resembling microfibrils whose synthesis has just commenced, are observable.

DISCUSSION
The involvement of lipid-linked oligosaccharide intermediates in cellulose synthesis is still a matter of debate despite numerous biochemical attempts to establish or disprove it (2, 21). The most widely quoted articles concerning the participation of lipid intermediates have been studies in vitro on bacterial cellulose (6, 10) and for the colorless chlorophycean algae Prototheca zopfii by Hopp et al. (8), but to date the topic is still controversial.

Based on the highly selective mode of action of TM (see introduction), the inhibition of microfibril deposition in O. solitaria autospores (Fig. 1b) strongly indicates that dolichol-(pyro)phosphate-bound saccharides play a role in the synthesis of cellulose in this algae. The concentration of TM administered, 10 μM, is in the range of that found to block almost completely in experiments in vitro the transfer of N-acetylglucosamine to dolicholphosphate (11). TM apparently does not interfere with energy metabolism or the availability of ATP (data not shown), although it cannot be excluded that other metabolic processes are unaffected. Unfortunately, in experiments in vitro, it has not yet been possible to demonstrate any enzymic activity of the dolicholphosphate pathway in isolated microsomal membrane fractions of O. solitaria (L. Lehle and H. Quader, unpublished observations). This may be due to the high shearing forces (French press) necessary to disintegrate the cells which may be harmful to the integrity of the whole membrane or the enzymes in question.

TM may affect microfibril assembly and deposition in two ways: first, by interfering with the transfer of cytosolic glucose to the cell surface via dolichol-linked intermediates; or second, by preventing the formation of an important glycoprotein. The occurrence of short microfibril imprints after the algae were treated with TM for 3 h or longer (Fig. 2c) seems to support the first notion by indicating that the microfibrils in synthesis could not be finished because the substrate supply is blocked by TM. Against this assumption, however, is the observation that dolicholphosphate-glucose synthesis is found to be associated predominantly with membrane fractions of low density and which coincide with the activities of ER-marker enzymes (13). What is the fate of the TCs? They could have been detached from the microfibrils and degraded or broken down into small fragments, which then might not be distinguishable from other particulate membrane components. A fragmentation of the TCs has been observed after Congo red treatment (15).

On the other hand, TM may prevent the synthesis of a glycoprotein and thereby possibly the intracellular transport of an unglycosylated polypeptide as shown to occur in several systems (4). With respect to O. solitaria, it is already known that microfibril deposition depends on continuous protein synthesis. Each microfibril layer apparently needs its own set of TCs (16, 19). TM apparently has no or only a minor effect on polypeptide synthesis judged from [3H]leucine incorporation studies but may prevent the formation of a glycoprotein essential for the synthesis of cellulose in O. solitaria.

α-Amylase secretion in barley aleurone cells is inhibited by TM (22). The authors discuss the possibility that the nonglycosylated form of α-amylase may be degraded faster than the glycosylated protein. In yeast, however, it has been shown that, as a result of the inhibition of the glycosylation, the synthesis of the polypeptide is also blocked without affecting the synthesis of other proteins (7). Similar mechanisms may be at play in Oocystis. Future investigations will have to examine these questions.

The TCs observed after short TM treatment (Fig. 2b) must have been synthesized and inserted into the plasma membrane shortly before the time of TM application.

According to Hopp et al. (8), the final step in the sequence forming cellulose precursors in Prototheca is the transfer of the oligo-glucose moiety from dolicholphosphate to a polypeptide resulting in a glycoprotein. What is the function of such a glycoprotein in cellulose synthesis? MacLachlan (12) has discussed the possibility that, analogous to the synthesis of other polysaccharides, a primer may be necessary. The idea that a glycoprotein may function as a primer for cellulose synthesis is highly intriguing. Franz (5) has presented the best evidence for this by demonstrating that, in vitro, a membrane fraction from Phaseolus, besides producing other products, also synthesizes a glycoprotein of which the saccharide part contains β-1,4-linked glycosides. It seems imaginable that the regularly arranged particles of a TC in the O. solitaria membrane may contain the glyco-(gluco)-protein in question.

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