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Cyclic Diguanylic Acid and Cellulose Synthesis in *Agrobacterium tumefaciens*

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The occurrence of the novel regulatory nucleotide bis(3′,5′)-cyclic diguanylic acid (c-di-GMP) and its relation to cellulose biogenesis in the plant pathogen *Agrobacterium tumefaciens* was studied. c-di-GMP was detected in acid extracts of ^{32}P-labeled cells grown in various media, and an enzyme responsible for its formation from GTP was found to be present in cell-free preparations. Cellulose synthesis in vivo was quantitatively assessed with [^{14}C]glucose as a tracer. The organism produced cellulose during growth in the absence of plant cells, and this capacity was retained in resting cells. Synthesis of a cellulosic product from UDP-glucose in vitro with membrane preparations was markedly stimulated by c-di-GMP and its precursor GTP and was further enhanced by Ca^{2+}. The calcium effect was attributed to inhibition of a c-di-GMP-degrading enzyme shown to be present in the cellulose synthase-containing membranes.

Infection of dictyledonous plants with the pathogen *Agrobacterium tumefaciens* results in the formation of tumors called crown galls. The mechanism of tumorigenesis is unique in that it involves the transfer of genetic information from the bacterium to the plant cells. An initial step in the infection process is the site-specific attachment of the bacteria to the plant host cells (8). During this attachment, the bacteria synthesize cellulose fibrils that cover the surface of the plant cells (12). It has been proposed that the major role of cellulose synthesis by *A. tumefaciens* is the firm irreversible anchoring of a large number of bacteria to the host cells, thereby aiding the production of tumors (10, 12). The present study was aimed at elucidating the enzymatic mechanisms and regulation of cellulose synthesis in *A. tumefaciens* and its relationship to the newly discovered regulatory nucleotide bis(3′,5′)-cyclic diguanylic acid (c-di-GMP). This nucleotide, enzymatically formed from GTP, plays a central regulatory role in modulating the rate of cellulose synthesis in another cellulose-producing bacterium, *Acetobacter xylinum* (15–17), and there is some evidence that UDP-glucose: β-glucan synthase activity in cell-free preparations of *A. tumefaciens* is affected by exogenously added c-di-GMP (20). We hereby report on the natural occurrence of c-di-GMP in *A. tumefaciens* together with enzyme systems responsible for its formation and degradation and on marked stimulation of cellulose synthase activity by the cyclic dinucleotide and its precursor GTP.

MATERIALS AND METHODS

Chemicals. All radiochemicals were from the Radiochemical Centre (Amersham, England). c-di-GMP, [^{32}P]c-di-GMP, and [terminal 5′-^{32}P]pG3′p5′G were prepared as previously described (16). Thin-layer plates of polyethyleneimine (PEI)-cellulose were from Machery Nagel (polygram Cel 300 PEI). Purified exocellobiohydrolase from *Trichoderma reesei* was a gift from Sharon Shoemaker (Cetus Corporation, Emeryville, Calif.).

Cells. Nopaline-producing *A. tumefaciens* C58 containing the pGV3851 Ti plasmid (23) (a gift of H. Zosnak, Faculty of Agriculture, Hebrew University) was grown in glass-distilled water in a Gyrotory shaker at 28°C on medium YEB (0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% sucrose, and 2 mM $MgSO_4$). Cell concentration was estimated from total cell protein (assumed to compose 50% of cell dry weight) which was determined by the method of Lowry et al. (9) after alkali digestion of the cells.

Enzyme preparation. All operations described below were performed at 0 to 4°C. Cells grown for 24 h were washed by centrifugation in 50 mM Tris hydrochloride (pH 7.5) containing 10 mM $MgCl_2$ and 1 mM EDTA (TME buffer) and suspended in the same buffer containing 20% (wt/vol) polyethylene glycol 4000. Cells at a concentration of 25 mg (dry weight) per ml were ruptured in a French pressure cell at 20,000 Db/in^2. The extract was centrifuged at 12,000 × *g* for 10 min, and the pellet containing the membrane fraction was suspended with a Teflon homogenizer in TME buffer. The suspension was recentrifuged at 1,500 × *g* for 3 min to remove large particulate material. The supernatant comprises the unwashed membranes. This fraction was recentrifuged at 18,000 × *g* for 20 min. The resultant pellet resuspended in TME buffer comprises the washed membranes, typically containing 5 to 6 mg of protein per ml. The supernatant fraction obtained is referred to as supernatant of washed membranes and typically contained 4 to 5 mg of protein per ml.

^{32}P labeling and extraction of cells. For extraction of intracellular nucleotides, cells were grown in the following media: medium A, 40 mM MOPS (morpholinepropanesulfonic acid), 4 mM Tricine buffer (pH 7.2) (Sigma Chemical Co., St. Louis, Mo.), 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.2 mM KH_2PO_4, 0.5 mM $MgSO_4$, 0.01 mM $FeSO_4$, 1% glucose (13); medium B, 0.2% Casamino Acids (Difco Laboratories, Detroit, Mich.), 0.066% yeast extract, 0.9% NaCl, 0.01% $MgSO_4$, 0.001% $FeSO_4$, 0.3% glucose; medium C, 0.09% Casamino Acids, 0.03% yeast extract, 0.18 glycerol (7).

Starter cultures grown separately on the various media were diluted 50-fold into 3 ml of fresh medium supplemented with 100 μCi of carrier-free $^{32}P_i$. Identical parallel cultures lacking $^{32}P_i$ were inoculated and monitored for growth. The cultures were grown with shaking at 28°C. When growth reached a cell density of 0.2 to 0.5 mg (dry weight) per ml, the radioactive cultures were rapidly mixed with 0.1 volume

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of cold 11 N formic acid, shaken in the cold for 30 min to extract nucleotides, and centrifuged for 5 min at 10,000 × g. The supernatant extract was used for chromatographic analysis.

TLC. Ion-exchange thin-layer chromatography (TLC) was performed on PEI-cellulose plates (20 by 20 cm) prewashed with 0.5 M LiCl, rinsed with distilled water, and dried before use. Two-dimensional chromatography for resolution of nucleotides was done as described by Bochner and Ames (4), employing 0.75 M Tris–0.45 N HCl–0.5 M LiCl (solvent A) for the first dimension and 1.5 M KH_2PO_4 (pH 3.65) (16) (solvent B) for the second dimension. Other solvents used in this work were 0.2 M NH_4HCO_3 (solvent C), 0.2 M $(NH_4)_2CO_3$ (solvent D), and 5.5 M $(NH_4)SO_4$ (pH 3.5) (solvent E). Labeled compounds were detected by autoradiography; the exposure time varied from 4 h to 1 day. For quantitation, the radioactive spots were cut out from the plate and counted in toluene-based scintillation fluid. When indicated, radioactive spots were scraped off and eluted with 0.5 M $(NH_4)_2CO_3$. The eluates were desalted by lyophilization and then dissolved in water.

Determination of UDP-glucose substrate depletion. Reaction mixtures (0.2 ml) containing 70 mM Tris hydrochloride (pH 8.6), 10 mM $MgCl_2$, 0.9 mM EDTA, 20 μM UDP-[U-^{14}C]glucose, and 0.4 mg of protein of unwashed or washed membranes were incubated for 15 min at 30°C and then deproteinized by heating at 100°C for 3 min and centrifugation. Samples of the resultant supernatant were applied to PEI-cellulose plates and resolved in 0.2 M KH_2PO_4 (pH 4.0). The ^{14}C-labeled UDP-glucose spot was detected by autoradiography, excised, and counted. Percent degradation was determined by comparison with control reaction mixtures which were boiled for 3 min before the addition of UDP-glucose. More than 80% of the original amount of UDP-glucose added was recovered from the reaction mixtures.

Enzymatic assay of c-di-GMP. The enzymatic assay of c-di-GMP is based on c-di-GMP activation of the cellulose synthase of *A. xylinum* (16, 17). This activation is highly specific for c-di-GMP. Other nucleotides tested (for a complete list, see Table 2 in reference 3) and the following guanosine derivatives could not substitute for c-di-GMP in stimulating synthase activity of washed membranes of *A. xylinum*, nor did their presence affect c-di-GMP stimulatory activity: GTP, GDP, GMP, guanosine-3′,5′-cyclic monophosphate, diguanosine tetra- or pentaphosphate, guanosine 3′-diphosphate-5′-diphosphate and guanosine 3′-diphosphate-5′-triphosphate (16). The activity of the enzyme in digitonin-solubilized washed membranes (prepared as described in reference 1) was determined by measuring the rate of incorporation of radioactivity from UDP-[^{14}C]glucose into alkali-insoluble 1,4-β-D-glucan. c-di-GMP is heat stable (16), and c-di-GMP-containing samples were deproteinized by heating at 100°C for 3 min followed by centrifugation. Samples of the resultant supernatant were added to standard assay mixtures made to contain (in a final volume of 0.2 ml) 70 mM Tris hydrochloride (pH 8.6), 10 mM $MgCl_2$, 5.0 mM $CaCl_2$, 0.9 mM EDTA, 20 μM UDP-[U-^{14}C]glucose (140 cpm/pmol), and digitonin-solubilized washed membranes of *A. xylinum* (0.3 mg of protein). The mixtures were incubated for 5 min at 30°C. The reactions were terminated, and the ^{14}C-labeled product formed was determined as previously described (2). The concentration of c-di-GMP in the sample was calculated by comparing the increase in cellulose synthase activity above basal activity observed in its presence with corresponding increases in synthase activity brought about by the addition of various concentrations of c-di-GMP (0.02 to 2.0 μM) (Fig. 1).

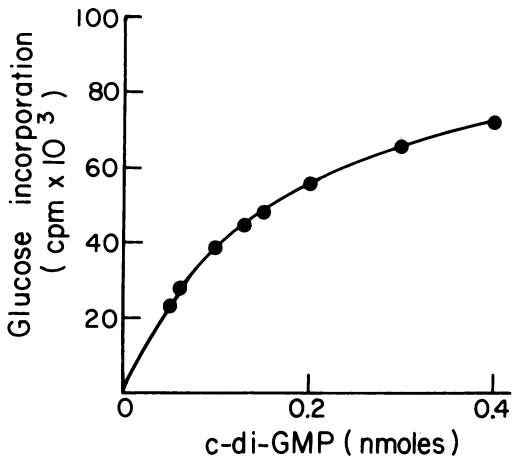

FIG. 1. Effect of added c-di-GMP on the activity of the cellulose synthase of *A. xylinum*. Digitonin-solubilized washed membranes of *A. xylinum* were assayed for cellulose synthase activity in the presence of increasing amounts of c-di-GMP as described in Materials and Methods. In the absence of c-di-GMP (basal activity), 1,000 cpm of ^{14}C-glucose were incorporated.

Radiochemical assay of c-di-GMP. The radiochemical assay of c-di-GMP is based on the isolation of [^{32}P]c-di-GMP enzymatically formed from [α-^{32}P]GTP. Reactions were terminated by heating the mixtures to 100°C for 3 min followed by centrifugation. Samples of the resultant supernatants were applied to PEI-cellulose plates and submitted to two-dimensional chromatography as described above. The radioactive spot on the plate corresponding to that of a similarly chromatographed [^{32}P]c-di-GMP marker was cut out and counted. The amount of c-di-GMP formed was calculated from total ^{32}P incorporation and the specific radioactivity of the GTP (2 mol of GTP per mol of c-di-GMP formed).

[^{14}C]glucose utilization in vivo. (i) Growing cells. Cells were grown for 24 h at 28°C with shaking in culture tubes containing 2 ml of medium YEB in which sucrose was substituted by 40 mM [U-^{14}C]glucose (680,000 cpm/μmol). The aggregate-containing cultures (total cell yield, 1.6 to 2.0 mg [dry weight]) were filtered onto Whatman GF/A fiber glass filters. The filters were washed with water until the filtrates were radioactive-free and then treated with 5 ml of 0.5 N NaOH at 100°C for 20 min. The mixtures were refiltered and washed consecutively with 4 ml of water (six times) and 4 ml of methanol (one time). The filters containing alkali-insoluble product were dried at 60°C and counted. When [^{14}C]glucose was added to a culture similarly grown on unlabeled glucose and the mixture was immediately filtered and treated as above, the radioactivity in the final product was negligible.

(ii) Resting cells. Cells grown for 24 h were washed in 0.05 M phosphate buffer (pH 6.0) and then suspended in the same buffer. Reaction mixtures (2.0 ml) in rubber-stoppered scintillation vials contained 50 mM phosphate buffer (pH 6.0), 40 mM [U-^{14}C]glucose (680,000 cpm/μmol), and 1 mg (dry weight) of cells. CO_2 formed was trapped in a small tube containing 0.5 ml of hyamine hydroxide (Packard Instrument Co., Inc., Rockville, Md.) which was placed in each vial. Mixtures were shaken at 30°C for the times indicated. Reactions were terminated by injection of 1 N HCl, and the

mixtures were then filtered, washed, alkali treated, dried, and counted as in part i above.

Analysis of glucan product. Total acid hydrolysis of the radioactive alkali-insoluble reaction products synthesized from [U-^{14}C]glucose by either growing or resting cells and from UDP-[U-^{14}C]glucose by membrane preparations in the presence of c-di-GMP and analysis of hydrolysis products were performed as previously described (1). Enzymatic digestion of the alkali-insoluble products was performed at 30°C for 2 h in 0.05 M sodium acetate buffer (pH 5.0) in the presence of a highly purified *Trichoderma* exocellobiohydrolase (0.4 mg/ml). This enzyme preparation displays a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and a single N-terminal amino acid (S. Shoemaker, personal communication). The enzyme has no detectable β-glucosidase activity and does not attack laminarin (1.3-β-glucan) and 1.2-β-glucan formed by extracts of *A. xylinum* (2), at least under the assay conditions employed here. After incubation, reaction mixtures were brought to 66% ethanol, chilled, and filtered onto Whatman GF/A glass fiber filters. The radioactivities of dried filters and a sample of the filtrate were counted to quantitate the extent of digestion. A portion of the filtrate from the enzymatic digest was concentrated and chromatographed on Whatman no. 4 paper with *n*-propanol–ethyl acetate–water (7:1:2) as the solvent. The chromatogram was divided into 1-cm strips and counted. Sugar markers (glucose, fructose, mannose, galactose, laminaribiose, sophorose, gentiobiose, and maltose) were detected by treatment with alkaline silver nitrate (21).

RESULTS

Occurrence of c-di-GMP in *A. tumefaciens*. Formic acid extracts of cells grown in the presence of $^{32}P_i$ in three different media, which differ with regard to carbon, nitrogen, and phosphorus sources, were analyzed by two-dimensional TLC for the presence of c-di-GMP. In all cases, autoradiography of the chromotograms revealed a well-resolved spot which coincided with that of a similarly chromotographed ^{32}P-labeled c-di-GMP marker (Fig. 2). Extracts of the spot were subjected to further TLC analysis in various solvent systems; in all cases, a single spot exhibiting the distinct mobility of c-di-GMP was detected (R_f values of 0.19, 0.35, and 0.03 in solvents C, D, and E, respectively).

Formation of c-di-GMP in cell-free preparations. The supernatant derived from washed membranes of *A. tumefaciens* catalyzed the conversion of GTP into a product identified as c-di-GMP by its chromatographic mobility and biological activity. In these experiments, the supernatant of washed membranes (0.15 to 0.30 mg of protein) was incubated for 10 min at 30°C in the presence of 50 mM Tris hydrochloride (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 3.0 mM $CaCl_2$, a GTP regeneration system (composed of 2 mM creatine phosphate and 4 U of creatine phosphate kinase), and either 0.2 mM [α-^{32}P]GTP (90,000 cpm/nmol) or 0.2 mM unlabeled GTP in a final volume of 0.2 ml. The reactions were terminated by heating to 100°C for 3 min followed by centrifugation. Samples of the deproteinized supernatants were assayed for c-di-GMP by the radiochemical assay and by the enzymatic assay as described in Materials and Methods. In experiment 1, 0.23 and 0.20 nmol of c-di-GMP were determined by the radiochemical and enzymatic assays, respectively. In experiment 2, 0.39 and 0.45 nmol of c-di-GMP, respectively, were found. In the standard enzymatic assay, 0.05 ml of the deproteinized supernatants from experiments 1 and 2 increased cellulose synthase activity 26- and 39-fold, respectively, above basal activity. After incubation with [α-^{32}P]GTP, analysis of heat-deproteinized reaction mixtures by two-dimensional TLC revealed that label was incorporated into a spot which cochromatographed with a [^{32}P]c-di-GMP standard. Similarly, heat-deproteinized preparations previously preincubated with unlabeled GTP stimulated the c-di-GMP-dependent cellulose synthase derived from *A. xylinum*; the extent of stimulation was linearly dependent on the amount of supernatant fraction preincubated with GTP and on the time of preincubation up to 20 min. The amount of c-di-GMP formed as estimated from label incorporation was consistent with the results derived from measurements of stimulatory activity. c-di-GMP formation did not occur with a supernatant fraction preheated for 3 min at 100°C.

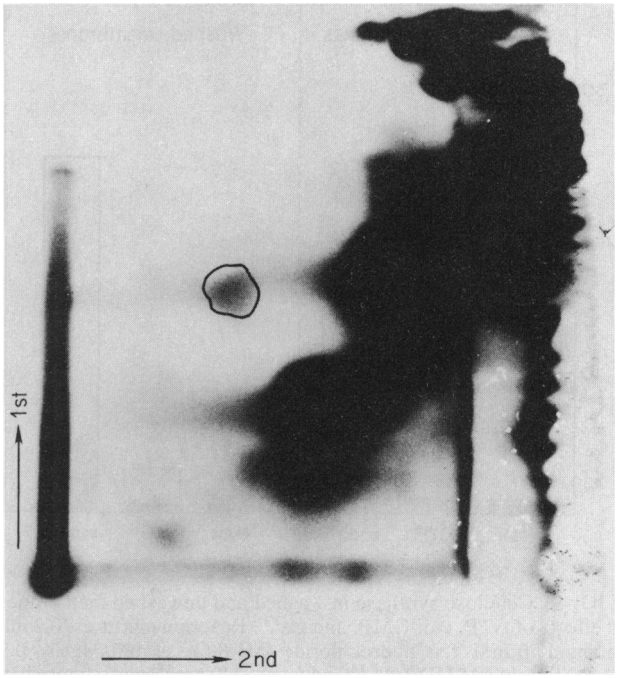

FIG. 2. Two-dimensional TLC separation of intracellular c-di-GMP in ^{32}P-labeled extracts of *A. tumefaciens*. Formic acid extracts of cells grown in the presence of $^{32}P_i$ were chromatographed in solvent A in the first dimension and in solvent B in the second dimension as described in Materials and Methods. Presented is an autoradiogram exposed from a two-dimensional separation of a P_i-labeled extract derived from cells grown on medium A. The dark line shows the superimposed position located by autoradiography of a [^{32}P]c-di-GMP standard chromatographed with an unlabeled extract.

Cellulose synthesis in growing and resting cells of *A. tumefaciens*. Cells grown in the presence of [U-^{14}C]glucose incorporated radioactivity into an alkali-insoluble product which was characterized as 1,4-β-D-glucan (cellulose) as described below. A similar product was formed by washed nonproliferating cells of *A. tumefaciens* upon incubation with [U-^{14}C]glucose. The rate of cellulose synthesis by resting cells, which was linear for up to 3 h, was similar to the average rate of cellulose synthesis by growing cells. Cells were grown for 24 h or incubated for 0.5, 1.0, 2.0, and 3 h in the presence of [U-^{14}C]glucose, and the products formed were analyzed as described in Materials and Methods. Growing cells converted 31 nmol of glucose to cellulose per h per mg of cells. Resting cells converted 28 nmol of glucose into cellulose and 980 nmol of glucose to CO_2 per h per mg of cells (calculated

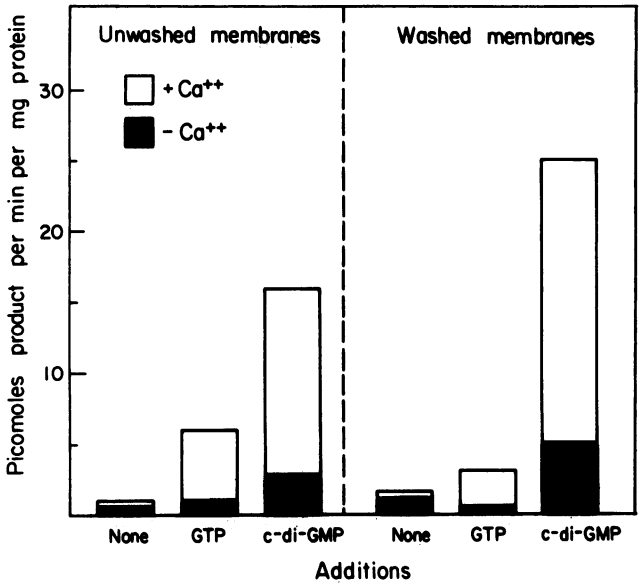

FIG. 3. Cellulose synthase in washed and unwashed membranes and effects of GTP, c-di-GMP, and Ca^{2+}. Reaction mixtures (0.2 ml) contained 70 mM Tris hydrochloride (pH 8.6), 10 mM $MgCl_2$, 0.9 mM EDTA, 20 μM UDP-[U-^{14}C]glucose (140 cpm/pmol), unwashed or washed membranes (0.4 mg of protein), and, as shown, 0.25 mM GTP, 5 μM c-di-GMP, or 5 mM $CaCl_2$. Incubation time was 15 min at 30°C. The reactions were terminated, and the alkali-insoluble ^{14}C-labeled product formed was determined as described previously (2). In control experiments with membranes preheated for 3 min at 100°C, no radioactivity could be detected in the alkali-insoluble fraction.

from total ^{14}C incorporation and the specific radioactivity of glucose). Cellulose formation by resting cells was accompanied by extensive substrate oxidation, and the molar ratio of glucose polymerized to that converted to CO_2 was 0.03.

Total acid hydrolysis of the labeled product derived from both growing and resting cells converted more than 90% of the radioactivity into free [^{14}C]glucose. The alkali-insoluble product was 95% solubilized upon treatment with a highly purified exocellobiohydrolase; chromatography of the enzyme hydrolysate showed that 80% of the solubilized radioactivity cochromatographed with a cellobiose standard, which was clearly separated from laminaribiose, sophorose, and gentiobiose standards. The remainder of the radioactivity cochromatographed with a glucose standard.

UDP-glucose:1,4-β-D-glucan 4-β-D-glucosyltransferase (cellulose synthase) in cell-free preparations of *A. tumefaciens* and its activation by GTP and c-di-GMP. Membrane preparations derived from extracts of *A. tumefaciens* catalyzed the transfer of glucose from UDP-glucose to an alkali-insoluble product characterized as 1,4-β-D-glucan as described above. This activity was not detected in supernatant fractions derived from cell extracts or from washed membranes. With unwashed membranes prepared in buffer containing polyethylene glycol 4000, cellulose synthase activity was markedly stimulated by both GTP and c-di-GMP. On the other hand, in membranes washed in buffer lacking polyethylene glycol, the enzyme was only slightly activated by GTP but still displayed a marked stimulation of activity by c-di-GMP (Fig. 3). These stimulatory effects cannot be attributed to inhibition of substrate depletory reactions, since more than 80% of the initial UDP-glucose could be recovered from unsupplemented reaction mixtures as judged by chromatography

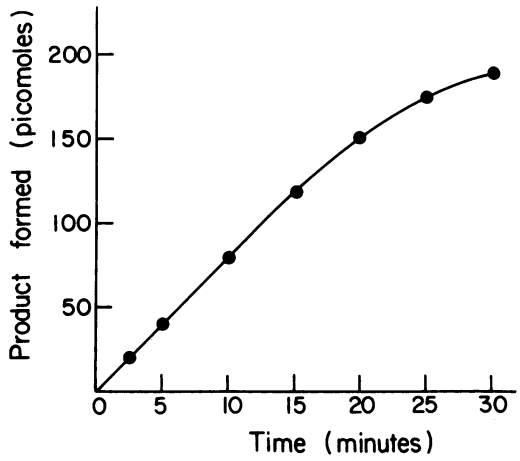

FIG. 4. Time course of cellulose synthase activity by washed membranes. Conditions were as described in the legend to Fig. 3 with washed membranes (0.4 mg of protein), 5 μM c-di-GMP, and 5 mM $CaCl_2$ except that the time of reaction was varied as indicated.

analysis (see Materials and Methods). Reactions were linear with respect to time for 15 to 20 min (Fig. 4) and were linear with respect to enzyme concentrations with washed membranes in the assay up to 0.5 mg of protein (Fig. 5). The reaction had an absolute requirement for Mg^{2+}. Optimal activity occurred at approximately 10 mM Mg^{2+}. In the absence of added Mg^{2+}, activity was completely inhibited by 0.2 mM EDTA. The synthase displayed normal Michaelis-Menten kinetics with respect to the substrate UDP-glucose with an apparent K_m of 0.15 mM (Fig. 6).

Degradation of c-di-GMP by Ca^{2+}-sensitive membrane enzyme. Stimulation of synthase activity by either GTP or c-di-GMP was greatly enhanced by the presence of Ca^{2+} (Fig. 3). This is probably related to the presence in the synthase-containing membranes of an enzyme capable of rapidly degrading c-di-GMP. The activity of this enzyme was heat sensitive, absolutely dependent on Mg^{2+}, and inhibited by Ca^{2+} (Fig. 7). c-di-GMP degradation was linear with time and with the amount of membrane preparations (Fig. 8). The phosphate-containing products arising from ^{32}P-labeled c-di-GMP upon incubation with washed membranes were

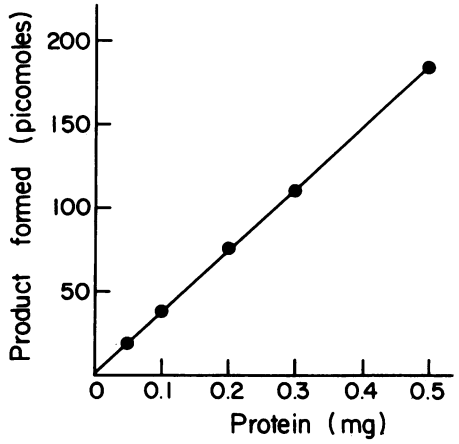

FIG. 5. Cellulose synthase reaction by washed membranes as a function of protein concentration. Conditions were as described in the legend to Fig. 4, except that the protein concentration was varied as indicated. Reaction time, 15 min.

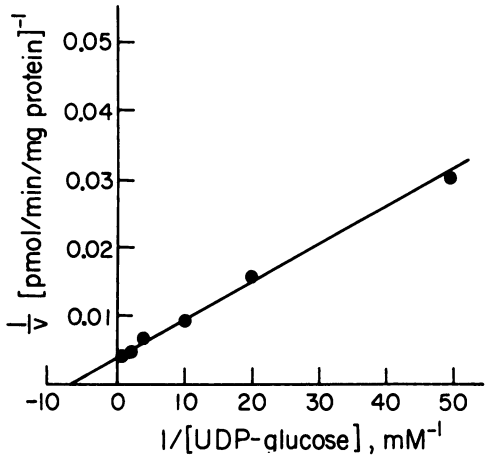

FIG. 6. Double-reciprocal plot of cellulose synthase activity versus UDP-glucose concentration. Conditions were as described in the legend to Fig. 4, except that UDP-[^{14}C]glucose concentration was varied. Reaction time, 15 min.

identified by their mobility in TLC as pG3'p5'G, 5'-GMP, and P_i (Fig. 9). Considering the structure of c-di-GMP and the occurrence of phosphomonoesterase activity in the membranes employed, it appears most likely that the action of this enzyme on 5'-GMP is responsible for the inorganic phosphate formed.

DISCUSSION

In this report, we demonstrated the in vivo occurrence of c-di-GMP in *A. tumefaciens*, the formation and degradation of the compound by in vitro preparations, and the stimulation by c-di-GMP of cellulose synthesis by membrane preparations from this bacterium. The presence of c-di-GMP in *A. tumefaciens* was indicated by the isolation from cells grown on different media of a compound chromatographically indistinguishable from c-di-GMP. A similar compound

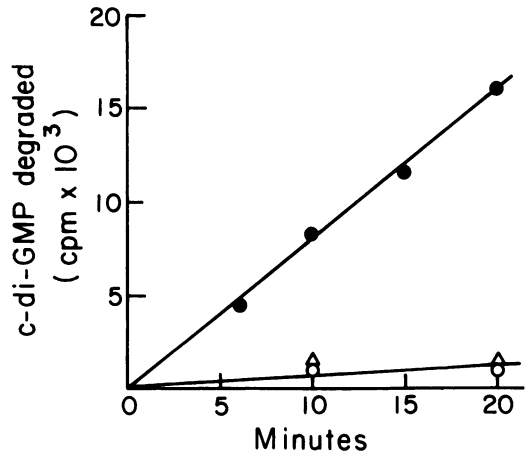

FIG. 7. Time course of c-di-GMP degradation by washed membranes. Reaction mixtures (0.2 ml) contained Mg^{2+}-free washed membranes (prepared by centrifuging a regular preparation in TME buffer and suspending it in 50 mM Tris hydrochloride [pH 7.5]), 0.4 mg of protein, 15 μM [^{32}P]c-di-GMP (80,000 cpm/nmol), 50 mM Tris hydrochloride (pH 7.5), and 0.9 mM EDTA. Symbols: ●, 10 mM $MgCl_2$; △, 10 mM $MgCl_2$ plus 5 mM $CaCl_2$; ○, no addition. Reactions at 30°C were stopped at the times indicated by heating at 100°C for 2 min followed by centrifugation. Samples (20 μl) of the deproteinized supernatants were applied to PEI-cellulose plates developed in solvent B. The region corresponding to c-di-GMP was excised and its radioactivity was counted. c-di-GMP degradation at a zero time control was negligible.

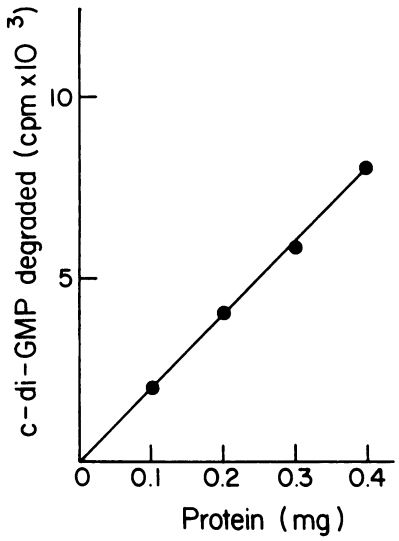

FIG. 8. c-di-GMP degradation by washed membranes as a function of protein concentration. Reaction mixtures (0.2 ml) contained 50 mM Tris hydrochloride (pH 7.5), 10 mM $MgCl_2$, 0.9 mM EDTA, 10 μM [^{32}P]c-di-GMP (80,000 cpm/nmol), and washed membrane protein as indicated. Reaction time was 10 min at 30°C. Reactions were terminated and c-di-GMP degradation was assayed as described in the legend to Fig. 7.

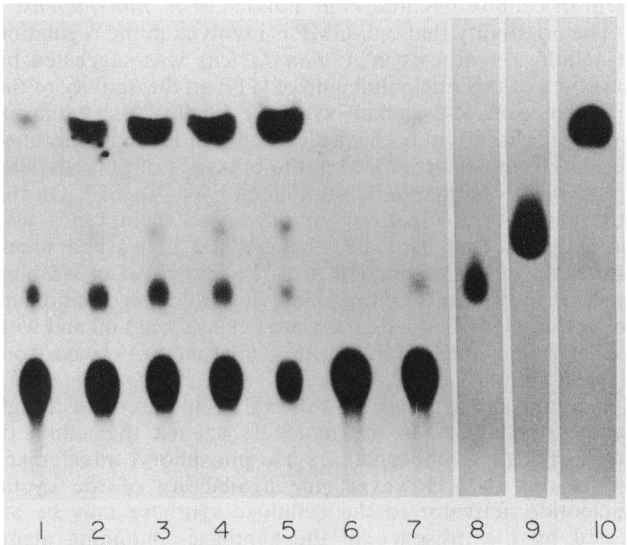

FIG. 9. Products of c-di-GMP degradation by washed membranes. [^{32}P]c-di-GMP (10 μM) was incubated with washed membranes (0.1 mg of protein) at 30°C in a final volume of 0.2 ml containing 50 mM Tris hydrochloride (pH 7.5), 10 mM $MgCl_2$, and 0.9 mM EDTA. At the times indicated, samples of the reaction mixture were boiled for 2 min, centrifuged, and analyzed by TLC. Presented is an autoradiogram of a PEI-cellulose plate developed in solvent B. Lanes 1 to 5, 2-, 5-, 10-, 20-, and 40-min reaction times. Lane 6, [^{32}P]c-di-GMP (zero time). Lane 7, 20 min in the presence of 5 mM $CaCl_2$. Lanes 8 to 10, ^{32}P-labeled markers: pG3'p5'G, 5'-GMP, and P_i, respectively.

was produced from GTP by a cell-free preparation. The identity of the product was confirmed by its ability to stimulate the c-di-GMP-dependent cellulose synthase of *A. xylinum*. Considering the high specificity of this activation toward c-di-GMP (16, 17), these results strongly suggest that the enzyme responsible for c-di-GMP formation, namely, diguanylate cyclase, is operative in *A. tumefaciens*.

The role of c-di-GMP in regulating cellulose synthesis in *A. xylinum* (17) offered the possibility that a probable target for c-di-GMP action in *A. tumefaciens* is its cellulose-synthesizing system. Cellulose synthesis in this organism was first reported by Deinema and Zevenhuizen (7) and later by Matthysse et al. (10–12) and Robertson et al. (14) in connection with its role in the attachment and binding of the bacteria to plant host cells. *Agrobacterium* species produces several β-linked glucans in addition to cellulose, including 1,2-β-glucan, 1,3,-β-glucan, and anionic β-glycans (19). In the present work, cellulose synthesis in vivo was studied and quantitatively assessed by measuring the rate of [^{14}C]glucose incorporation into a cellulosic alkali-insoluble product. The labeled product was characterized as a 1,4-β-glucan since it contained only glucose and was almost completely digested by a highly purified exocellobiohydrolase which does not attack 1,2- and 1,3-β-glucans. Moreover, when chromatographed, 80% of the radioactivity released by this enzyme migrated with cellobiose and the remaining radioactivity migrated with glucose but none migrated with the diglucosides contained within noncellulosic glucans known to be formed in *Agrobacterium* species (19). The results indicated that cellulose synthesis is an intrinsic property of *A. tumefaciens*. Thus, cells synthesized cellulose during growth in the absence of plant cells, and resting cells maintained a high capacity for cellulose synthesis. Considering the high cellulose-synthesizing capacity maintained in resting cells, these cells should be of practical value in the quantitative assessment of cellulose synthesis in mutants of *A. tumefaciens*.

The possibility that c-di-GMP is involved in the regulation of cellulose synthesis in *A. tumefaciens* was suggested by the effect of this nucleotide and of GTP on the activity of the UDP-glucose:1,4-β-glucan synthase (cellulose synthase). Synthase activity in both washed and unwashed membranes derived from cells ruptured in the presence of polyethylene glycol 4000 was markedly stimulated by c-di-GMP. On the other hand, while activity in unwashed membranes was similarly affected by GTP, the enzyme in washed membranes no longer responded to GTP. The effect of washing on GTP activation is compatible with GTP being the precursor of c-di-GMP in the diguanylate cyclase reaction and with the latter enzyme residing in the supernatant derived from washed membranes.

The unusual structure of c-di-GMP should confer a high degree of stability to the molecule against the action of broad-specificity phosphatases and phosphoryl transferases within the cell. However, the availability of the cyclic nucleotide activator to the cellulose synthase may be affected by the presence in the synthase-containing membranes of an enzyme(s) that rapidly degrades c-di-GMP into 5′-GMP, presumably acting as a phosphodiesterase. The overall pattern of degradation resembles that previously observed with membranous preparations of *A. xylinum* acting on c-di-GMP (17). Thus, degradation apparently occurs by two distinct steps, with the linear dimer pG3′p5′G as the initial product, and the degradation is inhibited by Ca^{2+} ions. This inhibition may account for the finding that c-di-GMP-induced stimulation of the membrane-bound cellulose synthase is markedly enhanced by Ca^{2+} (Fig. 3).

Taken together, the results presented here raise the possibility that *A. tumefaciens* possesses a regulatory system for cellulose synthesis similar to that described for *A. xylinum* (17). In this system, the target enzyme for regulation is the membrane-bound cellulose synthase and regulation is based on c-di-GMP and the enzymes involved in its synthesis and degradation (17). Although the two organisms share the overall capacity to polymerize glucose into 1,4-β-glucan and cellulose microfibril biogenesis, the ultrastructure of the final product assembled by each is significantly different. Whereas *A. tumefaciens* produces cellulose in the form of bundles and simple flocs (5, 7, 12), a more advanced mechanism of microfibril association is operative in *A. xylinum*, leading to the assembly of intricate composite ribbons and a pellicle of great strength and hydrophilicity (6). Furthermore, the two organisms markedly differ with respect to the efficiency of cellulose synthesis in terms of the molar ratio cellulose monomer to glucose oxidized to CO_2, which reflects the relationship between the polymerization process and overall cellular energy metabolism. In resting cells of *A. tumefaciens*, this ratio is rather low (0.03) compared with *A. xylinum*, which under similar conditions exhibits an efficiency ratio significantly greater than unity (18, 22).

Clarification of the physiological significance of the effect of c-di-GMP on in vitro cellulose synthesis and its relation to the role of cellulose synthesis in the anchoring of *A. tumefaciens* to plant cells should be facilitated through understanding the genetics of the biosynthetic process. Knowledge regarding the enzyme components of the system should assist the biochemical characterization of cellulose-negative mutants specifically deficient in regulatory or glucose-processing enzymes. Indeed, *A. tumefaciens* mutants with altered ability to synthesize cellulose fibrils have actually been isolated, including some having a cellulose-overproducing mutation (14). The occurrence of the regulatory nucleotide c-di-GMP in two different microorganisms raises the prospect that this compound may be of a more general natural occurrence and significance and warrants further research for its presence and role in other biological systems.

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