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GIBBERELLIN-CONTROLLED PECTINIC ACID AND PROTEIN SECRETION IN GROWING CELLS

STEPHEN C. FRY*

Botanical Laboratories, University of Leicester, Leicester, LE1 7RH, U.K.

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Key Word Index—*Spinacia oleracea;* Chenopodiaceae; spinach; suspension culture; gibberellic acid; growth; secretion; cell-wall components; pectinic acid; peroxidase; phenolic compounds.

Abstract—Gibberellic acid (GA) promoted cell expansion in a suspension culture of spinach. Preceding this response, weakly acidic pectinic acid was released into the medium. GA did not alter the concentration of sorbitol (0.7 M) needed to prevent growth, and sorbitol at this concentration did not block the action of GA upon the release of pectinic acid. GA suppressed the release into the medium of a peroxidase of MW *ca* 40 000 and favoured the intracellular accumulation of a polypeptide of similar MW. All the responses to GA were antagonized by 2,4-dichlorophenoxyacetic acid but not by kinetin. A hypothesis is presented in which the promotion of growth by GA is mediated by peroxidase, its phenolic substrates and the cell-wall pectic polysaccharides.

INTRODUCTION

Gibberellic acid (GA) strongly promotes the growth of certain plant tissues, probably by loosening the cell wall rather than by raising the turgor pressure [1]. This loosening must be due to a change in the structural components of the wall; the nature of the change is unknown and is discussed here.

A priori, GA-treatment could loosen the wall in 4 distinct ways: it could (a) promote the secretion from the protoplast into the wall of wall-loosening structural polymers, e.g. non-cellulosic polysaccharides; (b) inhibit the secretion of other structural polymers, e.g. hydroxyproline-rich protein [2] which may rigidify the wall; (c) promote the secretion of enzymes or co-factors, e.g. glycanases or H^+ [3], that catalyse wall-lysis; or (d) inhibit the secretion of wall-rigidifying catalysts, e.g. peroxidase [4]. It is hard to test these ideas in growing plant organs because it is difficult to isolate soluble cell-wall components free from cytoplasmic contamination. In contrast, in suspension cultures, the simple fully-defined liquid medium is a continuation of the cell wall; thus analysis of compounds released into the medium may give an indication of their concentration in the soluble phase of the wall matrix.

The view [5] that tissue cultures are not usefully GA-sensitive has recently been challenged and a suspension culture of spinach is promoted in growth by as little as 10 pM GA [6]. To investigate the mode of wall-loosening I have now studied the release of polymers into the medium during the promotion of growth in the spinach culture. Previous studies of the action of GA upon secretion have mainly dealt with non-growing systems such as the cereal aleurone layer.

RESULTS

Effect of GA on growth and the release of pectinic acid

GA at 10^{-11}–10^{-6} M promoted the expansion growth of spinach suspension cultures (Table 1 and [6]) but cell division was little affected [6]. Most of the cells remained isodiametric during the response to GA but the mean cell size was visibly increased.

The medium of GA-treated spinach cultures became turbid during several days' exponential growth whereas untreated controls remained clear. The following observations suggest that the turbid material was pectinic acid: (a) collected on a glass fibre paper disk, it failed to adsorb the fluorescent brightener Calcofluor White ST, suggesting the absence of cellulose; (b) it stained pink with ruthenium red, suggesting acidic groups; (c) it was insoluble in hot water or 3% sodium dodecyl sulphate or 0.1 M HCl, but dissolved readily in 0.1 M NaOH (cold) or 0.2 M EDTA (pH 6; hot); (d) it formed a jelly-like pellet upon high-speed centrifugation; (e) double hydrolysis of this pellet in H_2SO_4 yielded galacturonic acid (66%), arabinose (11%), galactose (7%) and rhamnose (8%) and only traces of glucose and xylose.

GA induced the release of soluble pectinic acid well before any turbidity appeared and before any promotion of growth (Fig. 1a). The release of soluble polymers containing uronic acid residues occurred principally 1–2 days after the addition of GA. The release of total polysaccharide occurred over a longer period but was unaffected by GA. The mean cell doubling time was *ca* 4 days.

*Present address: Department of Biochemistry, Unversity of Cambridge, Cambridge, CB2 1QW, U.K.

Table 1. Hormonal control of expansion growth (PCV) and the formation in the spent medium of a turbidity (A_{460}) due to pectinic acid in a suspension culture of spinach

Additives	PCV*	A_{460}	Additives	PCV*	A_{460}
None	31	0.006	GA_9 0.01 μM	30	0.010
GA_1 0.01 μM	55	0.091	1.00 μM	33	0.018
1.00 μM	60	0.100	GA_{13} 0.01 μM	31	0.007
GA_2 0.01 μM	34	0.017	1.00 μM	37	0.018
1.00 μM	45	0.063	2,4-D 5 μM	20	0.008
GA_3 0.01 μM	59	0.105	2,4-D 5 μM+GA_3 1 μM	18	0.010
1.00 μM	63	0.107	Kinetin 2 μM	34	0.006
GA_4 0.01 μM	33	0.010	Kinetin 2 μM+GA_3 1 μM	60	0.094
1.00 μM	40	0.021	Sorbitol 0.7 M	6	0.014
$GA_{4/7}$ 0.01 μM	44	0.029	Sorbitol 0.7 M+GA_3 1 μM	7	0.051
1.00 μM	57	0.106			

*PVC is expressed as μl packed cells per ml suspension culture. The cultures were harvested after 12 days' growth.

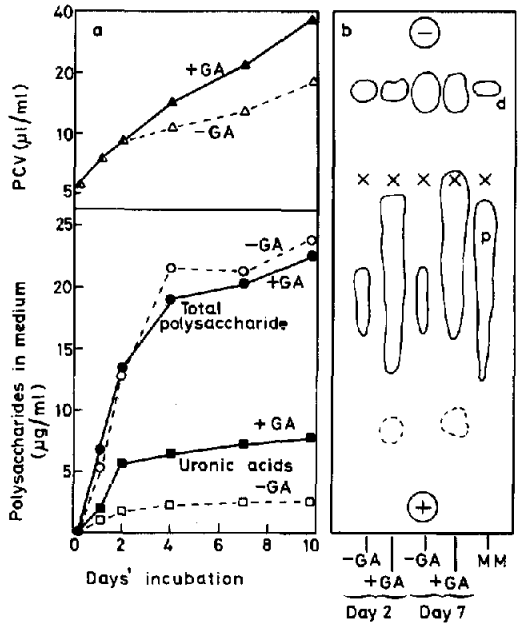

Fig. 1. Analysis of polysaccharides present in the spent medium of cultured spinach cells before a visible turbidity had appeared. Gibberellic acid, when present, was at 10^{-8} M. (a) Time-courses for growth (upper) and polysaccharide release (lower). (b) Drawing of a typical electrophoretogram of the soluble polysaccharides from the spent medium (−, cathode; +, anode; d, neutral dextran; X, origin; p, apple fruit weakly acidic pectinic acid; MM, marker mixture).

Pectic polysaccharides have been classified as neutral, weakly acidic or strongly acidic on the basis of their electrophoretic mobility on glass fibre paper at pH 6.5 [7]. Electrophoresis of the early released soluble polysaccharides revealed a neutral fraction (unaffected by GA) and a weakly acidic pectinic acid which was more abundant after GA-treatment; a trace of strongly acidic pectinic acid was also detectable after GA-treatment (Fig. 1b).

The effect of GA on pectic polysaccharides would be of limited interest if it only occurred in spinach. *Rosa* is taxonomically distant from *Spinacia* but it too is promoted in growth in suspension culture by GA [6]. During the response of *Rosa* the medium was again rendered turbid by a material with the properties of pectinic acid. GA-induced release of pectinic acid may thus be taxonomically widespread.

Hormonal specificity

The effects of GA (GA_3) upon growth and the long-term release of insoluble pectinic acid in spinach were mimicked by GAs 1, 2, 4, 7, 9 and 13 although the various gibberellins differed in effectiveness (Table 1). The auxin 2,4-D and the cytokinin kinetin did not promote either growth or pectinic acid release, and the presence of 2,4-D abolished the effectiveness of GA (Table 1). The release thus appears to be a gibberellin-specific response.

Relationship between growth and the release of pectinic acid

When growth was prevented with 0.7 M sorbitol, GA still induced the release of insoluble pectinic acid (Table 1). The release is thus not a result of growth and may be close to the primary action of GA. This notion is compatible with the time-course (Fig. 1a).

Further, GA-treatment appeared not to alter the concentration of sorbitol that was needed to prevent growth (Fig. 2). This suggests that the action of GA is

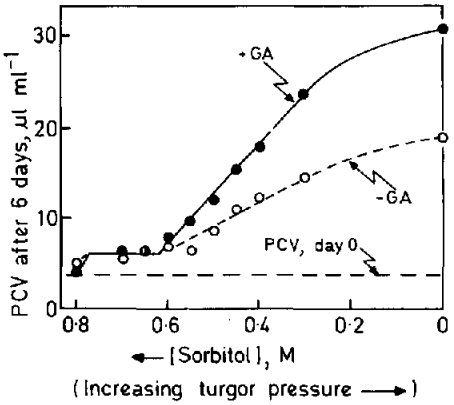

Fig. 2. The effects of sorbitol and GA (10^{-7} M) upon the growth of cultured spinach cells

Table 2. The effect of GA upon the peroxidase content and pH of the spent medium in a suspension culture of spinach

Parameter of spent medium	Value after 4 days' growth* −GA	+0.01 μM GA
Peroxidase activity (units/ml)	1.270±0.093	0.548±0.109
pH	4.82±0.03	4.95±0.05
Packed cell volume (μl/ml)	13.8±0.7	19.9±1.0

*Each value is the mean from 4 replicate cultures ± s.e.

not upon turgor pressure, and strengthens the view that cell-wall loosening (i.e. perhaps polysaccharide metabolism) could mediate the observed promotion of growth.

Acidity of spent medium

The short-term effect of auxins upon growth has been ascribed to an acidification of the water that permeates the cell wall [3]. Although the effects of GA in spinach cells reported here take place over days rather than minutes and are therefore not directly comparable with the acid-mediated action of auxins, it was considered worthwhile to examine the effect of GA upon the pH of the medium. GA caused the cells to render their medium slightly less acid (Table 2). This finding is evidence against the possibility that GA loosens the cell wall by the same mechanism as do auxins.

Protein secretion

In an earlier paper [4] it was reported that GA-treatment of cultured spinach cells suppresses the secretion of the haemoprotein peroxidase. This was confirmed in the present work (Table 2) and the kinetics of the response were determined (Fig. 3): the effect of GA on peroxidase appeared to precede slightly the effect on pectinic acid. All the gibberellins tested suppressed the net release of peroxidase activity although the action of GA_2 was rather weak (Table 3).

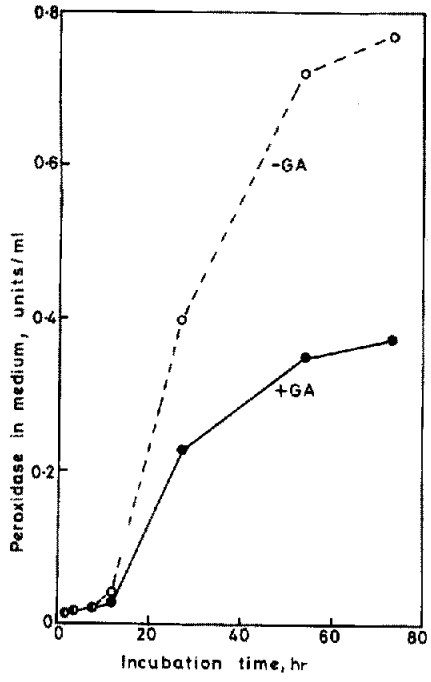

Fig. 3. The effect of GA (10^{-7} M) upon the release of peroxidase activity into the medium of cultured spinach cells.

Table 3. Hormonal control of expansion growth (PCV) and the peroxidase content of the spent medium in a suspension culture of spinach

Hormone	Measured values after 6 days' growth in the presence of hormone at 0.01 μM		hormone at 1.00 μM	
	PCV (μl/ml)	Peroxidase (units/ml)	PCV (μl/ml)	Peroxidase (units/ml)
None	26, 27, 27	2.96, 3.10, 2.75	–	–
GA_1	34	2.26 (−)	43	2.30 (−)
GA_2	27	2.96 (∘)	37	2.56 (∘)
GA_3	48	2.10 (−)	47	2.22 (−)
GA_4	29	2.66 (∘)	38	2.38 (−)
$GA_{4/7}$	31	2.60 (∘)	60	1.96 (− −)
GA_9	30	2.60 (∘)	33	2.14 (−)
GA_{13}	27	2.34 (−)	35	2.30 (−)
ABA	27	2.61 (∘)	28	3.08 (∘)
Benzyladenine	29	2.90 (∘)	32	2.42 (∘)
Kinetin	31	3.46 (+)	30	2.96 (∘)
2,4-D	28	4.60 (++)	23	4.82 (++)
NAA	30	3.32 (∘)	26	4.18 (++)

Symbols in parentheses categorize deviation from the untreated controls: (∘) = little effect, (+) = increase, (++) = strong increase, (−) = decrease, (− −) = strong decrease. 2,4-D = 2,4-dichlorophenoxyacetic acid; NAA = naphthaleneacetic acid.

Cytokinins and ABA had little effect; auxins promoted peroxidase release (Table 3) and were thus gibberellin-antagonists in this response as well as in the pectinic acid response (Table 1). (Kinetin, benzyladenine and ABA at the very high concentration of 10^{-5} M reduced not only peroxidase release but also growth and viability; data not shown.)

Analysis of the spent medium revealed 3 polypeptides (MWs 36 000–40 000) which were present at lower concentration in GA-treated spinach cultures than in untreated controls (Fig. 4a). The MW of these polypeptides was similar to that of spinach peroxidase (MW *ca* 40 000) as determined under non-denaturing conditions by chromatography on Sephadex G-150 [4]. In addition, GA-treatment favoured the intracellular accumulation of a major polypeptide (Fig. 4b). Its MW was *ca* 40 000 which again is compatible with its being peroxidase. The GA-induced accumulation of this polypeptide was prevented by the simultaneous presence of 2,4-D whereas kinetin was without effect (Fig. 4b).

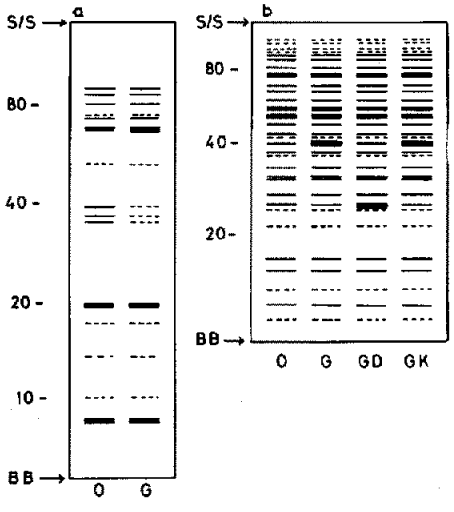

Fig. 4. Analysis of polypeptides present in (a) the spent medium and (b) the cells of suspension cultures of spinach grown for 7 days under various hormone regimes: O = no hormone; G = 0.01 μM gibberellic acid; GD = 0.01 μM GA + 5 μM 2,4-D; GK = 0.01 μM GA + 2 μM kinetin. The numbers give estimated MW ($\times 10^{-3}$). The thickness of the bands indicates their approximate intensity of staining with Coomassie blue; dashed bands were very faint. BB = Bromophenol blue marker; S/S = junction of stacking gel and separatory gel. (The 2,4-D induced polypeptide of MW *ca* 24 000 is unidentified.)

DISCUSSION

I recently suggested [4] that GA favours cell expansion by suppression of the secretion from the protoplast of a wall-rigidifying catalyst, namely peroxidase. I hypothesized that peroxidase stiffens the wall by catalysis of the oxidation of cell-wall phenolic compounds to form the more hydrophobic biphenyls, polymers or quinones, any of which could protect the wall polysaccharides against attack by enzymes or water. By suppression of peroxidase secretion, GA-treatment could maintain the phenols in their reduced state thus making the environment of the polymers less hydrophobic so that (i) covalently bound polysaccharides are rendered more susceptible to enzymic excision by existing hydrolases and transferases, (ii) hydrogen-bonded wall polysaccharides are freed owing to increased competition from water and (iii) growth is facilitated as a result of this loss of polysaccharides. Further, the GA-induced decrease in wall peroxidase content could hinder the covalent cross-linking of polysaccharides via diferuloyl bridges.

One prediction of the peroxidase hypothesis is that GA-treated cells ought to release polysaccharides, initially into the soluble phase of the wall matrix and finally into the culture medium, with which the matrix is continuous. Compatible with this prediction is the release of pectinic acid reported here. Perhaps a function of pectinic acid is to agglutinate the microfibrils of the cell walls in which it occurs; if so, then its GA-induced release would loosen these walls and favour cell expansion.

In intact plants there is no equivalent of the culture medium, but pectinic acid could still switch from a (covalently or non-covalently bound) form in which it contributes to the structural coherence of the wall to a (freely soluble) form in which it plays no structural role. Such a switch could again be mediated by changes in the oxidation state of the wall phenols, which could in turn be regulated by GA-controlled peroxidase secretion. In this way GA could promote the growth of whole plants by the same mechanism as proposed for suspension cultures. A solubilization of cell-wall pectic polysaccharides, required by the above suggestion, has been reported in ripening apple fruit [8], although this is a non-growing tissue and therefore not directly comparable.

It could be argued that it is not valid to liken suspension cultures to whole plants: stem tissue growth is promoted within 1 hr of GA-treatment [9, 10], i.e. much sooner than spinach suspension culture growth. It is possible that the early response of stems to both GA [11] and auxin [3] is mediated by a promotion of acid secretion into the cell wall. Acid secretion, however, cannot account for the long-term effects of GA, which probably require changes in the synthesis of DNA [12, 13], RNA [14–17], proteins [18–21], polysaccharides [22–27] and phospholipids [28, 29]. Any early acid secretion induced in the suspension culture by GA would probably be ineffectual because (a) the medium is already at pH 4–5, which is optimal for growth [30] and (b) the cell walls are well irrigated from a large volume of medium. The suspension culture may therefore provide a system in which it is possible to study purely the long-term (non acid-mediated) action of GA, and, to this extent, the culture could be a valid model for the whole plant.

Although the GA-induced release of pectinic acid into the liquid medium of spinach and rose cultures is compatible with the peroxidase hypothesis of post-synthetic modification of polysaccharides, the alternative hypothesis is equally tenable that GA acts to promote the synthesis of pectinic acid, some of which through over-production is lost to the medium. Intermediate hypotheses are also conceivable in which, for instance, the peroxidase acts within the endomembrane system before secretion into the cell wall: membrane phenols have been proposed to influence

membrane fusion [31], and the secretion of polymers could be a rate-limiting step in polymer synthesis [32].

Several workers have found that the addition of GA to plant material induces a decrease in the concentration of peroxidases [33–37], especially the cathodic isoenzymes [38], and this effect may cause a sparing of endogenous indole auxins [39]. However, little attempt has been made to detect effects of GA on the sub-cellular distribution of peroxidase. The present work verified that GA-treatment of cultured spinach cells suppresses the net release of peroxidase activity into the medium. Peroxidase-like polypeptides (MW *ca* 40 000) were also found in the medium and their behaviour paralleled that of the peroxidase activity. This suggests a control of enzyme levels rather than any modulation of enzymic action; the same conclusion was reached earlier when haemoprotein levels were measured [4]. The GA-induced suppression of the accumulation of peroxidase in the medium could be due to (i) inhibition of peroxidase synthesis, (ii) inhibition of its transfer through the plasmalemma, (iii) inhibition of its release from the cell wall, or (iv) promotion of its degradation or re-absorption. Model (iv) is unlikely because the peroxidase activity of the medium was never found to decrease, suggesting that it is rather stable. Model (iii) seems unlikely because it has been shown that the peroxidase content of cell walls closely parallels that of the culture medium [40]. If model (ii) is correct then GA ought to promote the intra-cellular accumulation of peroxidase: this prediction was apparently vindicated by the behaviour of a polypeptide of MW *ca* 40 000 (Fig. 4b). This polypeptide was not definitely identified; nevertheless, the quantity of it that accumulated in the cells in the presence of GA was in the same order of magnitude as that of peroxidase which accumulated in the medium in the absence of GA (rough calculations based on (a) the intensity of staining with Coomassie blue (Fig. 4b), (b) the quantity of haemoprotein released into the medium [4], and (c) unpublished biuret measurements of total cell protein per ml of culture). Further, the polypeptide of MW *ca* 40 000 does seem to be closely connected with the action of GA because it was the subject of one of five responses (increased cell expansion, increased release of pectinic acid, decreased release of peroxidase, increased levels of extra-cellular phenols [4] and increased intra-cellular accumulation of the polypeptide) that were all induced by GA and antagonized by 2,4-D but not kinetin. I therefore suggest that model (ii) is correct and a primary action of GA is the suppression of the transport of peroxidase into the cell wall; this is in line with the view [41] that phytohormones are intimately connected with membrane functions.

Two lines of published evidence are compatible with model (ii). Firstly, in dwarf maize leaves GA causes a cellular redistribution of Ca^{2+} [42], and Ca^{2+} has been shown to influence two phenomena that could regulate the secretion of peroxidase: (a) the binding of peroxidase to membranes [43], and (b) the fusion of Golgi vesicles with the plasmalemma *in vivo* [32] and *in vitro* [44]. Secondly, it has been asserted that peroxidase can form an inactive brown complex with phenolic substrates *in vitro* [45], and a similar brown complex seems to occur *in vivo* [46]. Thus if GA-treatment causes an intracellular build-up of peroxidase, a browning reaction might well occur within the cell, and such a reaction has often been seen in plant tissue cultures treated with GA [6, 47]. This observation, coupled with the fact that GA-treatment inhibits the accumulation of peroxidase in the medium, supports the possibility that GA acts through a suppression of the secretion of peroxidase.

EXPERIMENTAL

Tissue cultures. Green suspension culture line G10 [48] of spinach (*Spinacia oleracea* L. cv Monstrous Viroflay) was maintained as described before [4] in a medium lacking all hormones and vitamins and in which 1% sucrose and 0.02 mM EDTA were the sole organic additives. In the expts described here, exponentially growing cells were washed by repeated resuspension in sterile H_2O and then inocula (each 1 ml packed cell vol.) were transferred into 150 ml of routine medium [4] or this medium supplemented with phytohormones or sorbitol. The GAs were sterilized by filtration. Incubation was with shaking in the light at 20°, in 250 ml conical flasks sealed with Al foil [4]. A suspension culture of 'Paul's Scarlet' rose (*Rosa* sp.) was maintained by a slight modification [6] of a standard method [49]; the medium contained 2,4-D, kinetin and several vitamins. Expansion growth of both cultures was measured as packed cell vol. (PCV) after centrifugation of 15 ml of suspension at 1500 g for 5 min. Spent medium was obtained by filtration through 2 layers of 'Miracloth'.

Carbohydrate analysis. For hydrolysis, polysaccharides were dissolved in 72% H_2SO_4 with stirring at 25° for 3 hr, the acid was diluted to 3% and the soln was quickly autoclaved (120° for 1 hr). Hydrolysate was neutralized with $BaCO_3$ and the sugars were identified by descending PC on Whatman No. 1 paper in *n*-BuOH—C_6H_6—C_5H_5N—H_2O (5:1:3:3, upper phase) and in *n*-BuOH—HOAc—H_2O (15:3:5), stained with aniline hydrogen phthalate, eluted into 1% $SnCl_2$ in MeOH and estimated from A_{370} [50]. For quantitative analysis of soluble polysaccharides, spent medium was desalted on Sephadex G-25 and the high MW fraction assayed for total carbohydrate with sulphonated 1-naphthol [51] and for uronic acid residues with *o*-hydroxybiphenyl [52]. For qualitative analysis, further aliquots of the high MW fraction were subjected to glass fibre paper zone electrophoresis at 30 V/cm on Whatman GF/A paper in C_5H_5N—HOAc—H_2O (33:1:300), pH 6.5, containing 10 mM EDTA [7]; the polysaccharides were stained with sulphonated 1-naphthol in EtOH [7]. Neutral dextran and apple fruit weakly acidic pectinic acid [7] were used as markers.

Enzyme assay. The peroxidase soln was added to 10 vols. of a substrate soln containing 0.8 mM *o*-dianisidine, 0.8 mM H_2O_2 and 100 mM NaOAc buffer (pH 5), and monitored for A_{460} at 25° [53]. The enzyme soln contained 1 'unit/ml' if the coloured product accumulated at 1 A_{460}/min.

Polypeptide analysis. For analysis of polypeptides in the spent medium, the high MW fraction (see above) was concd under red. press and 5 vols. of PhOH—HOAc—H_2O (4:2:1, w/v/v) [54] were added. The suspension was centrifuged (12 000 g, 10 min, 25°) to pellet polysaccharides; protein was pptd from the supernatant by incubation with 5 vols. of Me_2CO at 0° for 2 hr. The ppt. was washed repeatedly with Me_2CO and the final pellet was dissolved in SDS/mercaptoethanol sample buffer [55] (100° for 10 min) and electrophoresed by a modification [56] of the method in ref. [55] using a 15% polyacrylamide/SDS gel. Staining was with Coomassie Brilliant Blue R. For analysis of

intracellular polypeptides the cells were rinsed free of medium, washed in warm 80% Me_2CO until chl-free, dried *in vacuo* and extracted with SDS/mercaptoethanol sample buffer [55] (50μl/mg cell dry wt) at 100° for 10 min. An aliquot (40 μl) was electrophoresed directly, as above but using an 11.7% gel.

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