Influences of developmental genes on localized glycogen deposition in colonies of a mycelial prokaryote, *Streptomyces coelicolor* A3(2): a possible interface between metabolism and morphogenesis

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SUMMARY

Two spatially localized phases of glycogen accumulation were detected by electron microscopy after cytological staining of thin sections of *Streptomyces coelicolor* A3(2) colonies. In phase I, glycogen granules were present in hyphae in the air–agar interface region of colonies that were undergoing aerial mycelium formation, though absent from aerial hyphae themselves. With one exception (a *bldF* mutant, which contained abundant glycogen), the absence of aerial mycelium caused by various developmental mutations (*bldA*, *bldB*, *bldC*, *bldD*, *bldG* and *bldH* mutations) was associated with a virtual absence of detectable glycogen. Mutations that allow aerial hyphae to form but prevent or interfere with the septation needed for spore formation (*whiA*, *whiB*, *whiG*, *whiH* and *whiI* mutations) did not impair phase I deposition. In phase II, abundant glycogen granules were present in aerial hyphal tips during intermediate stages of sporulation, but disappeared as spores matured. Phase II glycogen accumulation was observed with *bldA*, *bldC*, *bldD* and *bldG* mutants grown with mannitol as carbon source – conditions that allowed normal aerial mycelium development and sporulation; but phase I deposition was still at a very low level in these colonies. Glycogen was also deposited in the coiling tips of aerial hyphae of *whiA*, *whiB*, *whiH* and *whiI* mutants, and sporadic clusters of granules were present throughout *whiG* colonies. Significantly, glycogen was deposited in spore chains that developed ectopically in the normally spore- and glycogen-free substrate mycelium when multiple copies of *whiG* were present. Overall, the two phases of glycogen synthesis (and degradation) appear to be under separate developmental control rather than being mainly responsive to external growth conditions. Phase II glycogen levels were particularly high in a *whiE* mutant defective in spore pigment biosynthesis, and particularly low when hyper-pigmentation was induced by additional copies of the *whiE* genes. Spore pigment may therefore be a major sink for carbon stored as glycogen during sporulation. The possibility is discussed that, in addition to supplying carbon and energy at particular locations, glycogen synthesis and degradation may also play a part in morphogenesis by influencing turgor pressure.

1. INTRODUCTION

Macromolecules consisting of α1,4-linked glucose residues with varying amounts of α1,6 branches are ubiquitous in living organisms, in which they provide short- or long-term stores of readily available carbon. For example, in plants some of the carbon fixed during photosynthesis is converted to starch in chloroplasts, and sucrose derived from this primary storage reservoir is transported to longer-term storage organs such as tubers, and to developing seeds, where it re-forms starch in amyloplasts (Jenner 1982); and in mammals the muscular and circulating glucose levels are regulated by an elaborate protein kinase cascade acting to control the relative rates of glycogen synthesis and degradation (Cohen 1988). Many bacteria accumulate polysaccharide (usually glycogen) storage granules, particularly in conditions of nitrogen limitation and carbon excess (Preiss & Romeo 1989).

Streptomyces spp. are developmentally among the most complex of bacteria. The growth of a sporulating aerial mycelium on the branching substrate mycelium, and its temporal association with the production of diverse antibiotics and other secondary metabolites, has been subjected to analysis by molecular genetics, particularly in *Streptomyces coelicolor* A3(2) (reviewed recently by Champness & Chater 1994; Chater 1993). Glycogen has been detected in several *Streptomyces* spp. (*S. antibioticus*, *S. fluorescens*, *S. griseus* and *S. viridochromogenes*: Braña *et al.* 1982; *S. venezuelae*: Ramade & Vining 1992); and the differentiated organization of *Streptomyces* colonies has made it possible to see that glycogen accumulation is localized to two particular cell-types (Braña *et al.* 1980, 1986). A first phase of accumulation of glycogen (up to 20% of total cellular dry weight: Braña *et al.* 1986) takes place in hyphae in the region from which aerial branches emerge, and a second occurs in the tips of aerial hyphae undergoing sporulation.

These observations suggest that glycogen accumulation and degradation may play a part in morphological differentiation of streptomycetes (Braña *et*

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al. 1986; Chater 1989), and may even be under the control of cell-type-determining developmental regulatory genes. Recently, the nature of a few developmental regulatory genes of *S. coelicolor* A3(2) has been elucidated. One, *bldA*, which is required for both aerial mycelium formation and antibiotic production on normal (glucose-containing) minimal medium (Merrick 1976), specifies the *t*RNA for the codon *UUA* (Lawlor *et al.* 1987; Leskiw *et al.* 1991*a*). This codon is extremely rare in the *G*+*C*-rich genes of *Streptomyces* spp. (Wright & Bibb 1992) and it may well be largely confined to genes whose products are specifically required at the transition between vegetative growth and morphological differentiation, most likely in the substrate hyphae from which aerial hyphae emerge (Leskiw *et al.* 1991*b*). Such hyphae are one of the sites of glycogen accumulation. The bald colony phenotype (but usually not the antibiotic deficiency) of *bldA* and some other *bld* mutants can be phenotypically suppressed by growth on alternative carbon sources such as mannitol (Merrick 1976; Champness 1988) and it has been suggested that this might be due to a change in storage compound metabolism: the hypothesis was that osmotic pressure, a potentially important morphogenetic agent, might be influenced by the synthesis and degradation of storage compounds such as glycogen (Chater 1989). A second developmental gene, *whiG*, specifies an RNA polymerase sigma factor, σ^{WhiG}, that initiates sporulation in the tips of aerial hyphae (Chater *et al.* 1989), where the second phase of glycogen accumulation takes place. A third cloned gene, *whiB*, which is needed for the subdivision of aerial hyphal tips into unigenomic pre-spore compartments, encodes a small protein (WhiB) of a previously undescribed kind that may be a transcription factor (Davis & Chater 1992).

In this paper the observations of Braña *et al.* (1980, 1986) on glycogen synthesis in various *Streptomyces* spp. are extended to include *S. coelicolor* A3(2) and its close relative *S. lividans* 66, two organisms widely used in genetic studies (Hopwood *et al.* 1985), so that the effects on glycogen deposition of various genetic backgrounds that alter development could be assessed. The results suggest that *bldA* and several other *bld* genes are necessary for the first phase, but not the second, of glycogen accumulation, and that the second phase may depend on *whiG*.

2. MATERIALS AND METHODS

(*a*) *Bacterial strains*

S. coelicolor A3(2) strains examined included the wild-type and some morphological mutants derived directly from it [C85 (*whiA85*), C70 (*whiB70*), C71 (*whiG71*), C119 (*whiH119*) and C17 (*whiI17*) (Chater 1972)]; J1501 (*hisA1 uraA1 strA1* SCP1$^-$ SCP2$^-$ Pgl$^-$: Chater *et al.* 1982) and its constructed isogenic derivatives J1700 (with the *bldA39* mutation; Leskiw *et al.* 1991) and J1820 (with the *whiG71* mutation; Méndez & Chater 1987); and representative mutants in various *bld* genes [J669 (*bldB69*), J660 (*bldC18*) and J774 (*bldD53*), all from Merrick 1976; C103 (*bldG103*) and C109 (*bldH109*), both from Champness 1988; and 166 (*bldF*; Passantino *et al.* 1991): all these mutants also have auxotrophic mutations]. The *S. lividans* 66 derivatives 1326–9 (a β-galactosidase-deficient mutant: Eckhardt & Smith 1987) and its constructed isogenic derivative J1725 (with a *bldA39* mutation; Leskiw *et al.* 1991) were also examined. Observations were also made on *S. coelicolor* strains with introduced plasmids carrying *whiG* (pIJ4412, a high copy number plasmid also carrying *tsr*, conferring thiostrepton resistance: Méndez & Chater 1987), *whiG*-dependent promoters (pIJ4470, pIJ4471; both derivatives of the multicopy, *tsr*-containing, vector pIJ4083: Tan & Chater 1993; Clayton & Bibb 1990), or *whiE* ORFs I-VII (pIJ2156, a low copy number plasmid also carrying *tsr*: Davis & Chater, 1990).

(*b*) *Media and culture conditions*

All experiments used agar minimal medium (MM: Hopwood *et al.* 1985) with glucose (0.5% or, where specified, 2% w/v) or mannitol (0.5% w/v) as carbon source. The use of mannitol as carbon source in many experiments reflected observations that (i) aerial mycelium development of the wild-type is more reproducible and uniform with this carbon source than with glucose, and (ii) most classes of *bld* mutants defective in aerial mycelium on MM+glucose produce an apparently normal aerial mycelium on MM+-mannitol (Merrick 1976; Champness 1988). For auxotrophic strains, the medium was supplemented as in Hopwood *et al.* (1985). When plasmids were present, thiostrepton was added at 5 μg ml^{-1} unless stated otherwise. Incubation was at 30 °C. Cultures were grown either directly on the agar as isolated colonies, as in previous ultrastructural studies of developmental mutants (Wildermuth 1970; Wildermuth & Hopwood 1970; Hopwood *et al.* 1970; McVittie 1974), or from heavily seeded inocula on cellophane membranes or 0.45 μm pore-sized nitrocellulose filters (suppliers, Millipore; pre-sterilized by autoclaving between layers of filter paper) placed on agar base plates. The latter procedures allowed greater rapidity and uniformity of development, and made it easier to determine the localization of fields during electron microscopy; but note that the rate of development in such cultures depended on plating density, a parameter that cannot readily be standardized with morphological mutants because the plating units are various kinds of mycelial fragments. Samples were taken for electron microscopy at various times before and during sporulation.

(*c*) *Electron microscopy*

Specimens were prepared and examined as in Chater *et al.* (1989) except that for visualization of polysaccharides, here taken to be glycogen following the analysis of Braña *et al.* (1982), the sections were stained with periodic acid, thiocarbohydrazide and silver proteinate (Thiéry 1967; Robertson *et al.* 1975). Negative controls omitting any of these three reagents gave no pattern of electron-dense granules. More than one time-course of samples was generally examined.

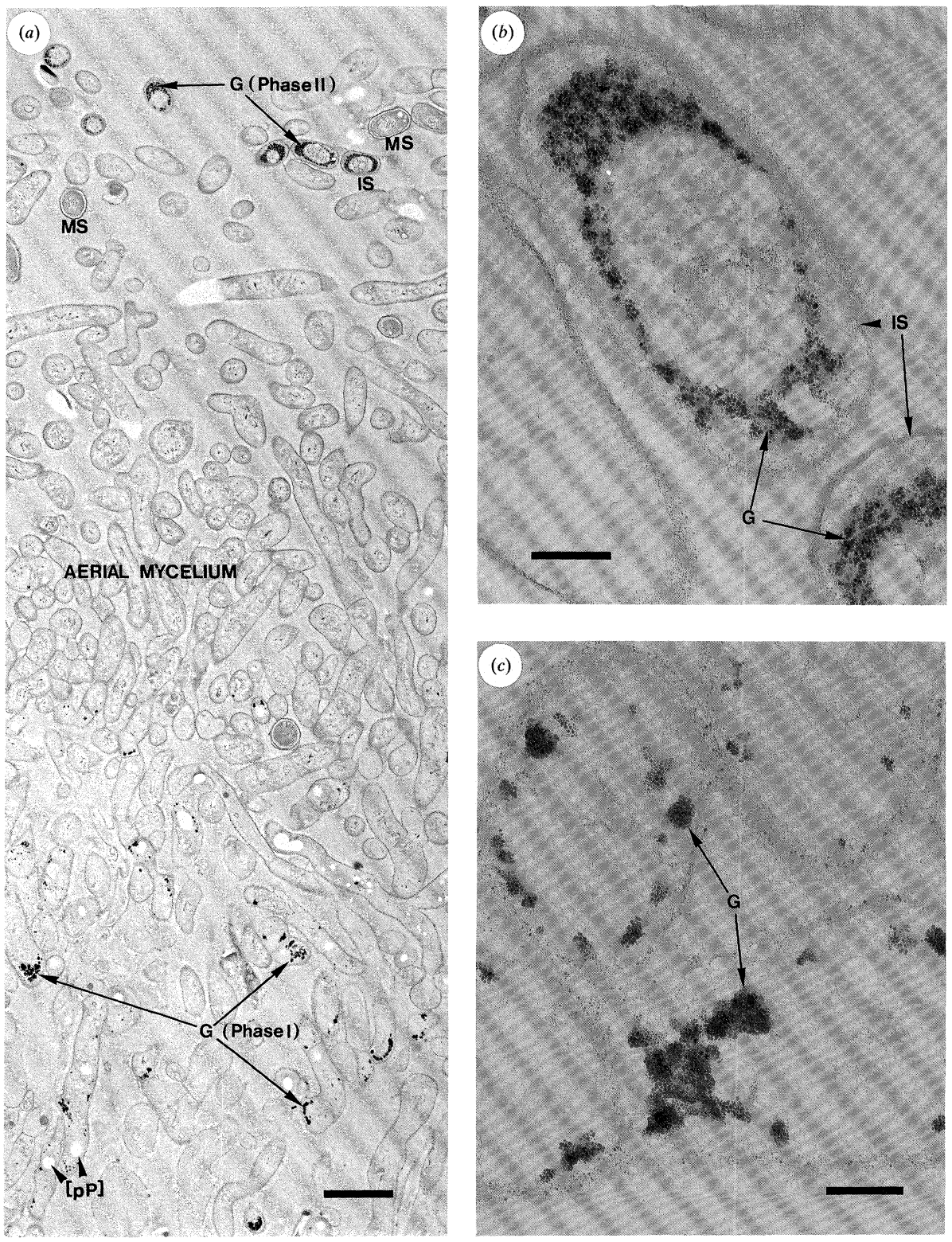

Figure 1. Two phases of glycogen synthesis in *S. coelicolor* A3(2). (*a*) Section extending from the upper surface to the centre of a colony. At a low abundance, glycogen granules are visible in the lower part of the mycelium (phase I). Glycogen is absent from aerial hyphae, but abundant in immature spore chains (phase II). As spores mature and develop thick walls, glycogen disappears. (*b*) Phase II, higher magnification. (*c*) Phase I, higher magnification. All sections were of an *S. coelicolor* A3(2) colony grown for 5 days directly on MM + mannitol. Key: G = glycogen; IS = immature spore; MS = mature spore; [pP] = hole left by destruction of polyphosphate granules. Bar markers: (*a*) 2 μm; (*b*, *c*) 200 nm.

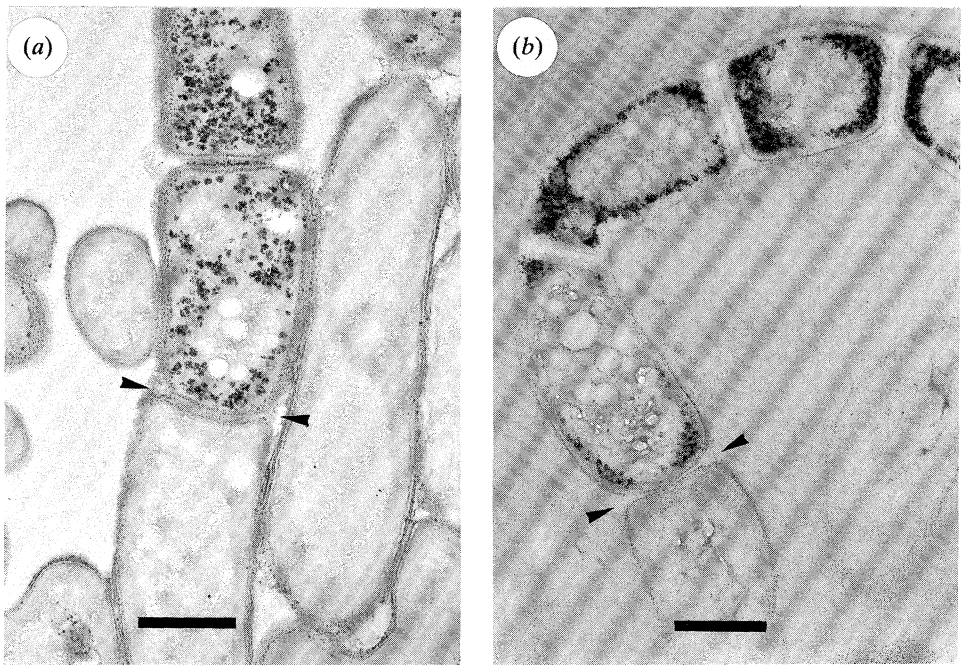

Figure 2. A sharp discontinuity of glycogen synthesis at the cross-walls (arrowed) separating pre-spore chains from their supporting aerial hyphae. Note also that the two faces of the arrowed cross-walls differ in the way in which they join the lateral wall (see text). (*a*) Strain J1501/pIJ486, MM plus mannitol, 4 days. (*b*) Strain A3(2), cellophane, MM plus 2% glucose, 5.75 days. Bar markers, 500 nm.

3. GLYCOGEN GRANULES ACCUMULATE AT TWO STAGES DURING COLONY DEVELOPMENT IN *STREPTOMYCES COELICOLOR* A3(2)

Previous studies of glycogen accumulation in *Streptomyces* spp. used strains that are little characterized genetically (Braña *et al.* 1980, 1982, 1986). However, genetic systems are available for *S. coelicolor* A3(2). Glycogen accumulation was therefore studied during development of this species using the silver proteinate procedure. The strains used were the A3(2) wild-type stain (from which many developmental mutants, including some used in this paper, were directly derived: Hopwood *et al.* 1970; Chater 1972) and strain J1501, a genetically marked, $^{\phi}$C31-sensitive, plasmid-free derivative of the A3(2) strain used here and elsewhere in the study of several cloned genes.

Irrespective of the major carbon source (i.e. 0.5% or 2% glucose, or 0.5% mannitol) strain A3(2) gave results similar to those previously described for *S. antibioticus* (Braña *et al.* 1986). Undifferentiated young cultures or the hyphae at the advancing edges of older colonies lacked obvious polysaccharide (glycogen) granules, but localized accumulation became apparent as aerial mycelium appeared. This accumulation (which we term here 'phase I') was confined to hyphae in a region from just above the surface of the agar to just below it, or to hyphae close to the supporting membranes (figure 1*a–c*). Somewhat more glycogen accumulated in this part of the mycelium on glucose (especially at 2%) than on mannitol. The A3(2) derivative J1501 showed less (though usually detectable) glycogen accumulation than did A3(2) itself at this stage. The non-sporulating hyphae in the aerial mycelium of colonies growing directly on agar were virtually free of granules in A3(2) or J1501.

When sporulation septation (Wildermuth & Hopwood 1970) began at the tips of aerial hyphae, a second phase (phase II) of glycogen accumulation was seen in A3(2) and J1501 (figure 1*a,b*; figure 2). This was confined spatially to those parts of aerial hyphae subdivided by sporulation septa, as shown most clearly in sections including the junction between the sporulating and non-sporulating parts of an aerial hypha: glycogen was present only in the sporulating part (figure 2). These striking discontinuities in glycogen deposition were also marked by a morphologically distinctive cross-wall between the hyphal stalk and the first spore compartment; the side of the wall in the compartment usually showed rounding of the junctions of the lateral and cross-walls, whereas a sharp ca. 90° angle was retained between these walls in the hyphal stalk compartment (figure 2). Phase II glycogen accumulation during sporulation was limited to the intermediate stages of sporulation (figure 1*a*): of 99 immature spore chains containing completed cross-walls, 86 contained glycogen, the proportion containing glycogen reaching a maximum just before spore wall thickening became apparent. Granules were seen in only 17% of more mature, thick-walled spores. There was no detectable glycogen in any of the three examples seen of incipient spore chains containing incomplete sporulation septa in this survey. In this respect, *S. coelicolor* seems to differ from *S. antibioticus*, in which glycogen is detectable before the sporulation septa are complete (Braña *et al.* 1980, 1986).

Silver-staining experiments carried out in parallel with *Streptomyces lividans* 1326–9 [which is closely related to *S. coelicolor* A3(2)] gave results like those with

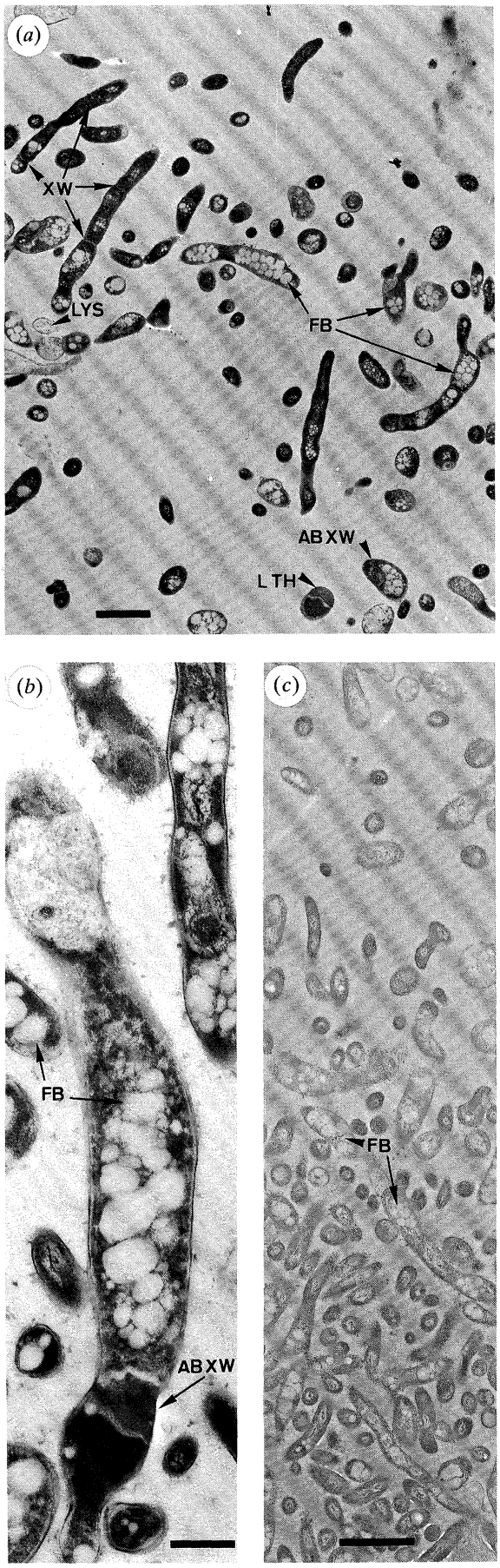

Figure 3. During growth on glucose, a *bldA* mutant makes no spores and accumulates little glycogen. (*a*) Uppermost cells of colony of J1700 (in this case containing the vector pIJ702), 5 days, uranyl acetate stain. (*b*) Higher magnification of part of (*a*). (*c*) J1700, uppermost cells, 7 days, silver proteinate stain (note absence of darkly stained glycogen granules). Key: ABXW = aberrant cross-wall; LTH = irregular lateral thickening of cell wall; LYS = lysed cell; XW = cross-wall; FB = fatty or oily bodies. Bar markers: (*a–c*) 2 μm; (*b*) 500 nm.

J1501; i.e. glycogen accumulated to a low level at phase I, and was obvious at phase II.

Within older cultures, circular holes and occasional electron-dense spherical bodies were seen in some hyphae and spore compartments (figure 1*a*). The dark bodies, which are not represented in any of the figures selected, appear to be polyphosphate deposits, and the white holes resulted from the destruction of the black bodies during processing or examination of specimens (K.A.P., R. Gowing and K.F.C., unpublished; Glauert & Brieger 1955; Hopwood & Glauert 1960). The spatial distribution of polyphosphate in wild-type colonies appeared broadly to coincide with that of glycogen.

4. A *bldA* MUTANT ACCUMULATES VERY FEW GLYCOGEN GRANULES WHEN GROWN IN CONDITIONS THAT SUPPRESS NORMAL MORPHOLOGICAL DEVELOPMENT

When growing on MM plus glucose, *bldA* mutants produce no recognizable aerial mycelium or spores, though they have been briefly reported to produce abundant crosswalls – perhaps related to sporulation septa – resulting in irregular mycelial fragments (Merrick 1976; Chater & Merrick 1979). If the two phases of glycogen accumulation are intimately associated with morphological differentiation, then *bldA* mutants might fail to accumulate glycogen on MM plus glucose. In uranyl acetate-stained sections of *bldA* mutants (J1700 of *S. coelicolor* or J1725 of *S. lividans*, with or without the plasmids pIJ702, pIJ486 or pIJ4083 which were used as vectors in experiments to be described below) the vegetative mycelium of young colonies was indistinguishable from that of the parental *bldA*$^+$ strain, but on glucose medium the mycelium at the 'bald' colony surface of the *bldA* mutants after several days was more pleomorphic, and there were no spores (figure 3*a*). Lysed or lysing cells, with generally electron-transparent contents devoid of morphological features, increased in number in these older samples (figure 3*a*). In some preparations many cross-walls were seen in these parts of the colonies, and these were irregular both in occurrence and in detailed morphology: there were often ingrowths from just one side of a hypha, and irregular thickening of the septa and of the cell wall was common (figure 3*a*,*b*). Many of the cells were of greater than normal diameter. Similar pleomorphic cells were also observed at a somewhat lower frequency in wild-type colonies. A conspicuous feature of the cytoplasm of these cells was the abundant occurrence of relatively electron-transparent areas that might be deposits of some fatty or oily storage compound (figure 3*a–c*). Very similar bodies observed in the wild-type under different growth conditions

contain triacyl glycerol (Olukoshi & Packter 1994; Olukoshi 1993).

Several independently prepared silver-stained samples of glucose-grown J1700 and J1725 were examined. Glycogen was seldom found (and only as low-abundance, very small granules) in these *bldA* mutants, whether in the central parts of the mycelium or close to the surface (figure 3*c*). Control cultures of the respective *bldA*$^+$ parental strains J1501 and 1326–9 showed deposits in both locations, though – as previously noted – only to a limited extent in the mycelium. Thus, all the comparisons suggested a sharply reduced ability of *bldA39* mutants to accumulate glycogen when grown on MM plus glucose. There was no reduction in the occurrence of polyphosphate-derived holes in *bldA* mutants.

5. GLYCOGEN IS PRESENT IN SPORE CHAINS PRODUCED BY A *bldA* MUTANT GROWN WITH MANNITOL AS CARBON SOURCE

When the *bldA* mutants of *S. coelicolor* or *S. lividans* were grown directly, or on nitrocellulose filters, on MM plus mannitol, spore-bearing aerial hyphae of apparently normal morphology were formed as briefly reported previously (Merrick 1976; figure 4*a*). Many of the sporulating chains contained abundant glycogen granules (figure 4*b*), whereas few granules were seen in non-sporulating hyphae (but somewhat more than with glucose as carbon source). The fraction of sporulating hyphae at each developmental stage, and the proportion at each stage containing glycogen granules, were in broad agreement with the observations for morphologically wild-type strains. The *bldA* mutants therefore appeared to be unconditionally impaired only in phase I glycogen synthesis, which was not restored by growth on mannitol. (J1700 cultures were also grown on cellophane on MM + mannitol, but in those conditions – and for unknown reasons – they did not produce aerial hyphae or spores, and resembled glucose-grown cultures in their ultrastructure and failure to produce glycogen.)

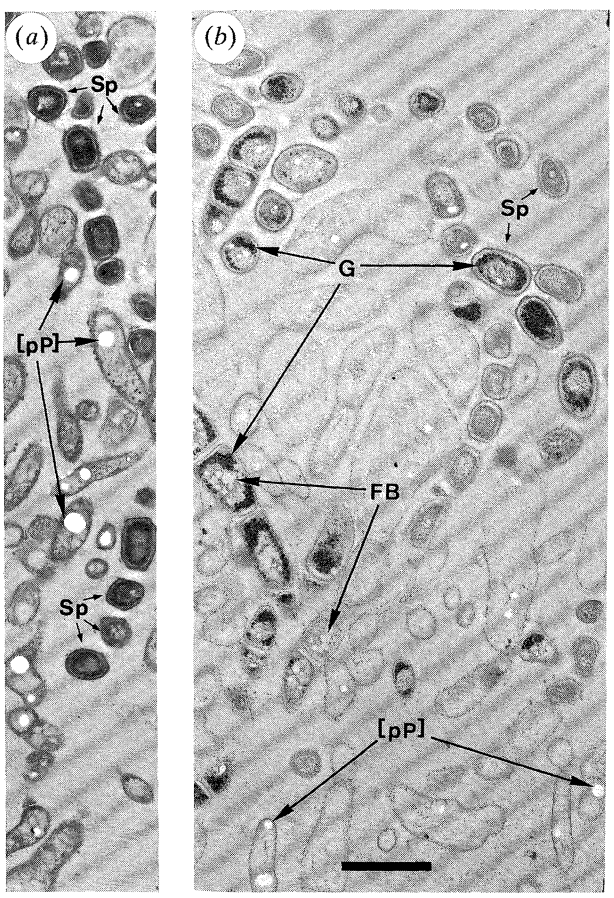

Figure 4. During growth on mannitol, a *bldA* mutant produces chains of spores showing phase II glycogen accumulation. (*a*) Upper layer of J1700, 5 days, uranyl acetate. (*b*) Upper layer of J1700, 7 days, silver proteinate. Key: Sp = spore or spore chain; [pP] = hole left by destruction of polyphosphate granule; G = glycogen; FB, fatty or oily bodies. Bar markers: 2 μm.

6. GLYCOGEN ACCUMULATION IN *bld* MUTANTS OF OTHER CLASSES

Various classes of *S. coelicolor* A3(2) *bld* mutants other than *bldA* have been described genetically, but there is little or no previous information available about their ultrastructure, nor about glycogen deposition in them. In the following descriptions, it should be borne in mind that the background genotypes of some of the strains are considerably diverged from the J1501 lineage.

The ultrastructure and silver-staining of various representative mutants are illustrated by figure 5 and summarized in table 1. The results reinforced earlier evidence of considerable phenotypic similarity between *bldA*, *bldD* and *bldG* mutants. In each case and in that of the *bldC* mutant, phase I glycogen deposition was greatly reduced compared with morphologically wild-type strains, and the aerial mycelial growth and associated sporulation induced when mannitol replaced glucose in the medium was accompanied by phase II glycogen deposition, albeit at relatively low levels in the *bldC* and *bldD* mutants. This strengthened the hypothesis that phase I deposition is developmentally regulated, and implicated *bldC*, *bldD* and *bldG* as well as *bldA* in this regulation; it also showed that phase I and phase II deposition are largely independently regulated. The *bldB* and *bldH* genes also seem to be needed for phase I glycogen deposition (but note that during these experiments we were unable to induce sporulation of the *bldH* mutant by growth on mannitol, even though this was possible in earlier studies: Champness 1988; B. Leskiw, personal communication). In contrast, the *bldF* mutant, the bald morphology of which was unaffected by the carbon source, showed no deficiency in glycogen deposition.

7. GLYCOGEN ACCUMULATION IN THE AERIAL HYPHAE OF MUTANTS DEFECTIVE IN THE FORMATION OF SPORULATION SEPTA

Phase II glycogen deposition may be part of the programmed development of spores. Several classes of *whi* mutants with blocks at different stages of sporu-

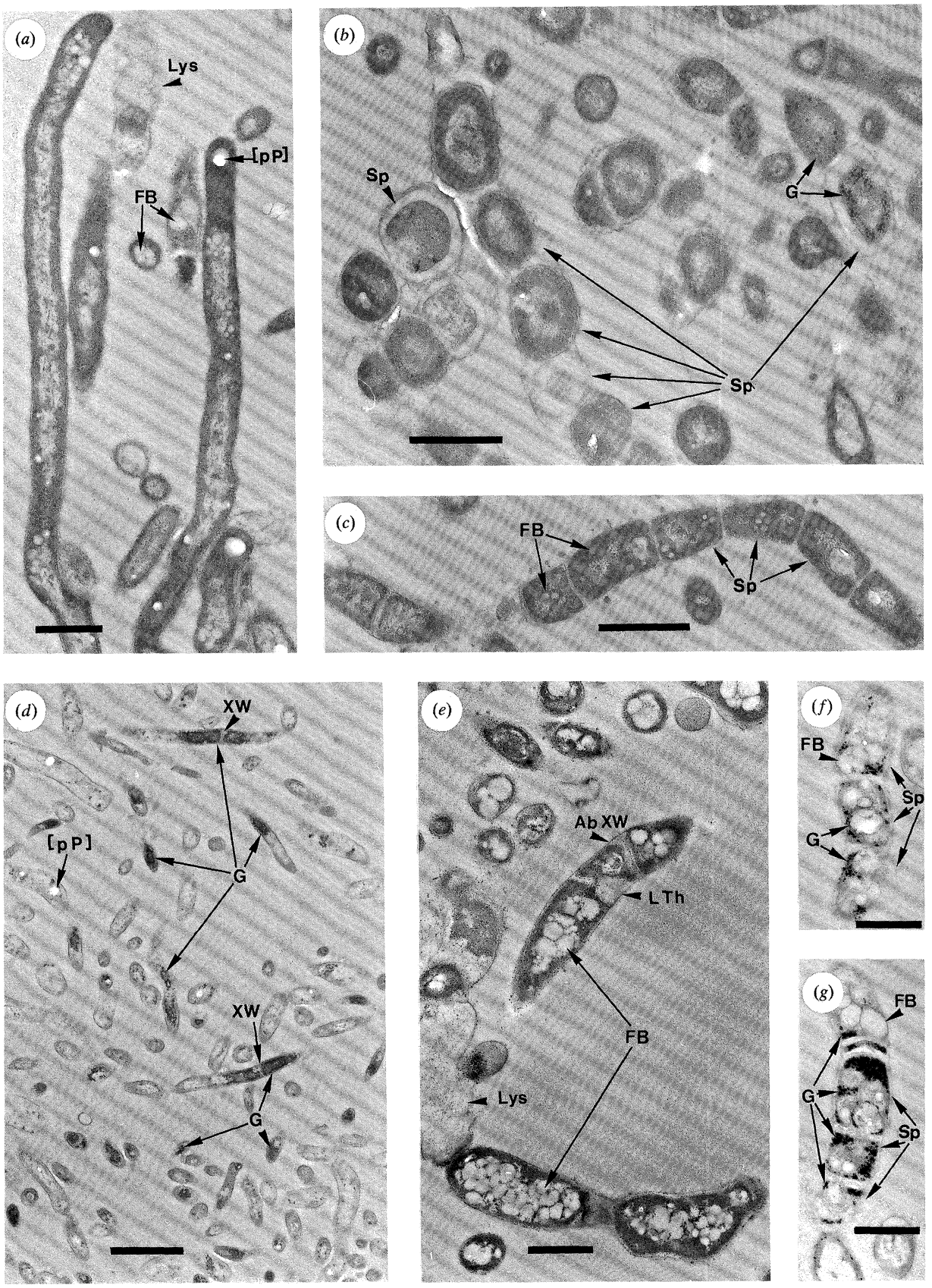

Figure 5. Glycogen accumulation in various representative *bld* mutants. All sections were stained with silver proteinate. (*a*) J669 (*bldB*), MM plus mannitol, 5 days: upper surface of culture; (*b*,*c*) J660 (*bldC*), MM plus mannitol, 5 days: (*b*), upper surface, (*c*), below the agar surface; (*d*) 166 (*bldF*), MM plus mannitol, 6 days: upper surface; (*e*) C103 (*bldG*), MM plus glucose, nitrocellulose, 45 h: upper surface; (*f*) and (*g*) C103 (*bldG*) MM plus mannitol, nitrocellulose, 45 h: aerial mycelium. Key: AbXW = aberrant crosswall; FB, fatty or oily bodies; G = glycogen; LTh = lateral thickening; Lys = lysed cell; [pP] = hole left by destruction of polyphosphate granule; Sp = spore; XW = crosswall. Bar markers: (*a*,*b*,*c*,*e*,*f*,*g*) 1 μm; (*d*) 2 μm.

Table 1. *Effects of bld mutations on glycogen accumulation*

relevant genotype (strain no.)	carbon source	morphology	glycogen deposition		illustration
			phase I	phase II	
wild-type (A(2), J1501 1326-9)	glucose	moderate aerial sporulation	+	++	figures 1,2
	mannitol	abundant aerial sporulation	+	++	figure 2
bldA39 (J1700, J1725)	glucose	bald	—[a]	NA[c]	figure 3
	mannitol	moderate aerial sporulation	—[a]	++	figure 4
bldB69 (J669)	glucose	bald	—	NA[c]	not shown
	mannitol	bald	—[a]	NA[c]	figure 5
bldC18 (J660)	glucose	bald	—	NA[c]	not shown
	mannitol	moderate aerial and ectopic sporulation	—	+(aerial) −(ectopic)	figure 5
bldD53 (J774)	glucose	bald	—	NA[c]	not shown
	mannitol	weak aerial sporulation	—	+	not shown
bldF (166)	glucose	bald	++	NA[c]	not shown
	mannitol	bald	++	NA[c]	figure 5
bldG103 (C103)	glucose	bald	—	NA[c]	figure 5
	mannitol	moderate aerial sporulation	—	++	figure 5
bldH109 (C109)	glucose	bald	—	NA[c]	not shown
	mannitol	bald[b]	—	NA[c]	not shown

[a] Small amounts occasionally found.
[b] In other reports, *bldH* mutants have been shown to produce a sporulating aerial mycelium on MM+Mannitol.
[c] NA, not applicable.

lation have been described (Hopwood *et al.* 1970; Chater 1972). Glycogen accumulation in representative *whi* mutant derivatives of strains A3(2) and J1501 was therefore examined, using colonies or cultures of various ages spanning the time at which the A3(2) and J1501 sporulating chains showed granules (figure 6). In confirmation of early morphological studies (Chater 1972; McVittie 1974) sporulation septa were absent from the *whiA*, *B*, *G* and *H* mutants, and seen only occasionally in the *whi*I mutant. In the *whiA*, *B*, *H* and *I* mutants some sections passed through the curled tips typical of aerial hyphae in these mutants, and some of these contained patches of glycogen granules (figure 6*a*, *b*, *d*, *e*), which were relatively dense in the *whiB* and *whiH* mutants. Thus, phase II glycogen synthesis does not seem to be severely dependent on *whiA*, *B*, *H* or *I*. In a survey of sections passing through well-defined curled aerial hyphae, the proportions containing silver deposits were: *whiA85* (C85) 7/22; *whiB70* (C70) 9/14; *whiI17* (C17) 2/11. A second *whiI* mutant (C225) was very similar to C17. The *whiH119* mutant (C119) showed an interesting dichotomy of cell types near aerial hyphal tips; some stained strongly with uranyl acetate and with silver proteinate, but the majority stained poorly with either. Phase I glycogen synthesis was more obvious in all of these *whi* mutants than in the wild-type controls.

A similar analysis of *whiG* mutants was hampered by the absence of obvious morphological features such as curling of aerial tips. Glycogen deposits were dispersed throughout the older parts of the upper half of colonies by three days, being maximally abundant at four to five days (figure 6*c*). There were fewer deposits close to the colony edge and the uppermost surface of the aerial mycelium than near the colony centre. The phenotype was compatible with, rather than strongly supportive of, the *whiG*-dependence of phase II glycogen deposition suggested in the next section.

8. ECTOPIC SPORULATION AND ASSOCIATED GLYCOGEN PRODUCTION INDUCED BY A HIGH COPY NUMBER OF *whiG* IN *bldA*$^+$ AND *bldA*$^-$ STRAINS

Growth (on MM plus mannitol) of *S. coelicolor* J1501 containing multiple copies of *whiG* (on the pIJ702-derived plasmid pIJ4412), either as isolated colonies

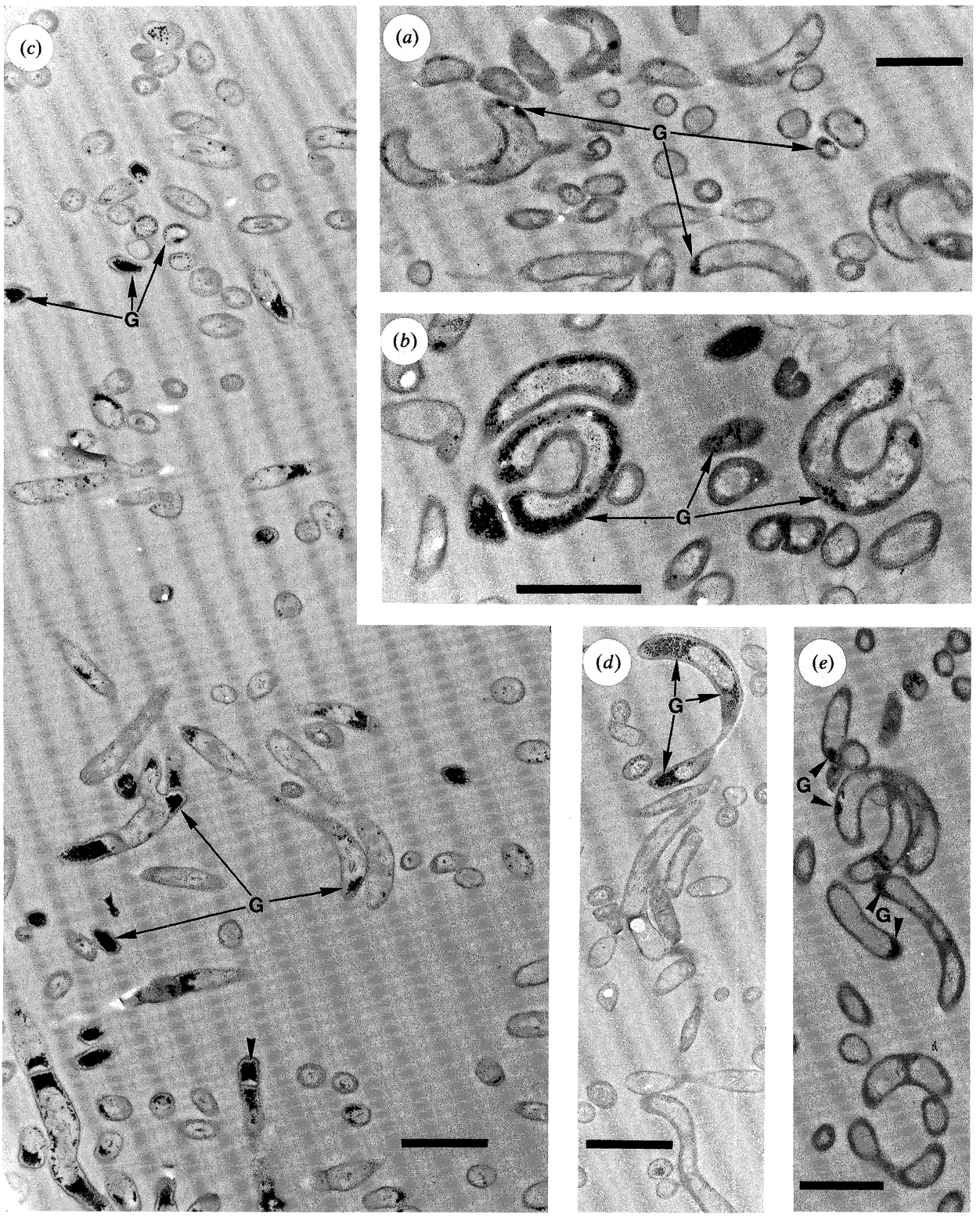

Figure 6. Glycogen deposition in the aerial hyphae of mutants (*whi*) defective in sporulation septation. All cultures were grown on mannitol and all sections were stained with silver proteinate. (*a*) C85 (*whiA85*), 4 days; (*b*) C70 (*whiB70*), 4 days; (*c*) C71 (*whiG71*), 5 days; (*d*) C119 (*whiH119*), 4 days; (*e*) C225 (*whiI225*), 4 days. Key: G = glycogen. Bar markers: 2 μm.

directly on the agar or as confluent areas of growth on nitrocellulose filters, resulted in extensive sporulation of both aerial and substrate hyphae, as previously briefly summarized (Méndez & Chater 1987; Chater *et al.* 1989). (Interestingly, multiple copies of *whiG* had little effect when glucose was the major carbon source.) This produced a situation in which the tightness of the association of phase II glycogen synthesis with sporulation could be further assessed. Although sporulation occurring ectopically in the substrate mycelium was often less regular than sporulation in the aerial mycelium, and individual spores were often misshapen,

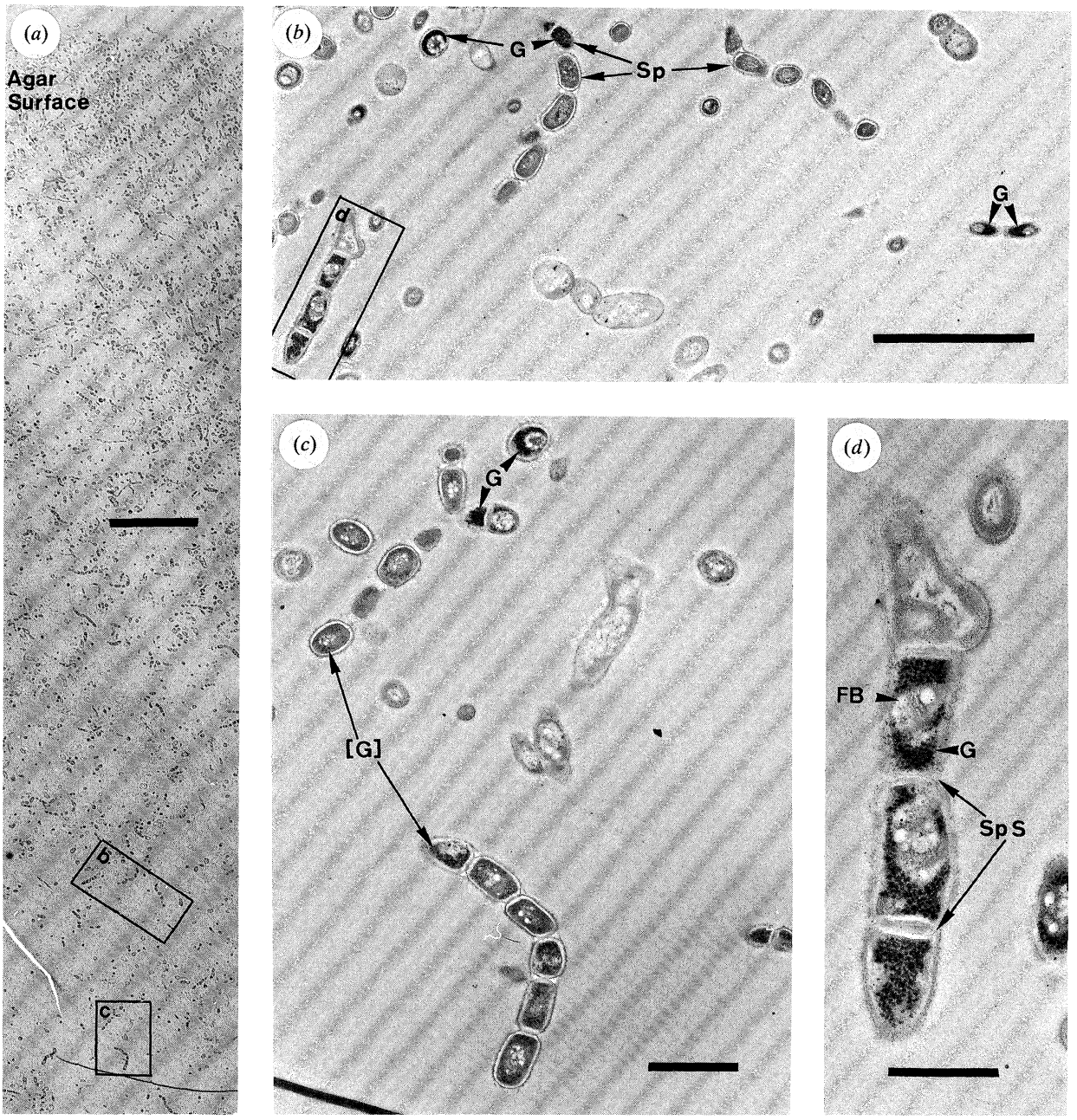

Figure 7. Glycogen synthesis in spore chains induced to develop ectopically by multiple copies of *whiG*. J1501 carrying pIJ4412 (a multicopy, *whiG*-containing plasmid) was cultured for 5 days on MM plus mannitol and stained with silver proteinate. (*a*) Vertical section through part of a colony. The position of the agar surface is indicated. Boxed areas containing spore chains are shown at higher magnification in (*b*), (*c*) and (*d*). Key: G = glycogen (brackets indicate light deposit); Sp = spore; SpS = sporulation septum; FB = fatty or oily bodies. Bar markers: (*a*) 20 μm; (*b*) 5 μm; (*c*) 2 μm; (*d*) 1 μm.

many examples could be found of sporulating chains in which the morphology and approximately regular spacing of the crosswalls that subdivided substrate hyphae into spore compartments was typical of specialized sporulation septa of *S. coelicolor* (Wildermuth & Hopwood 1970; McVittie 1974) (figure 7). The abundance of sporulation in J1501/pIJ4412 and its rapidity during growth on filters imply a moderate degree of synchrony. After 16 h, there was no evidence of sporulation septation, whereas at 22 h there were a few thick-walled spores among the many spore compartments. Thus the whole process of sporulation of aerial hyphae from initiation of septation to rounded, thick-walled spores, can take place in 6 h (or perhaps less).

Section 7 (above) addressed, somewhat inconclusively, the possibility that phase II of glycogen deposition could be under developmental control by sporulation regulatory genes rather than being a physiological response to nutrient limitation at aerial hyphal tips. If so, then *whiG*-induced ectopic sporulation in J1501/pIJ4412 grown on MM plus mannitol should also be accompanied by glycogen synthesis. Figure 7*b*, *c*, *d* verifies this expectation: glycogen granules were abundant in the ectopically sporulating parts of hyphae.

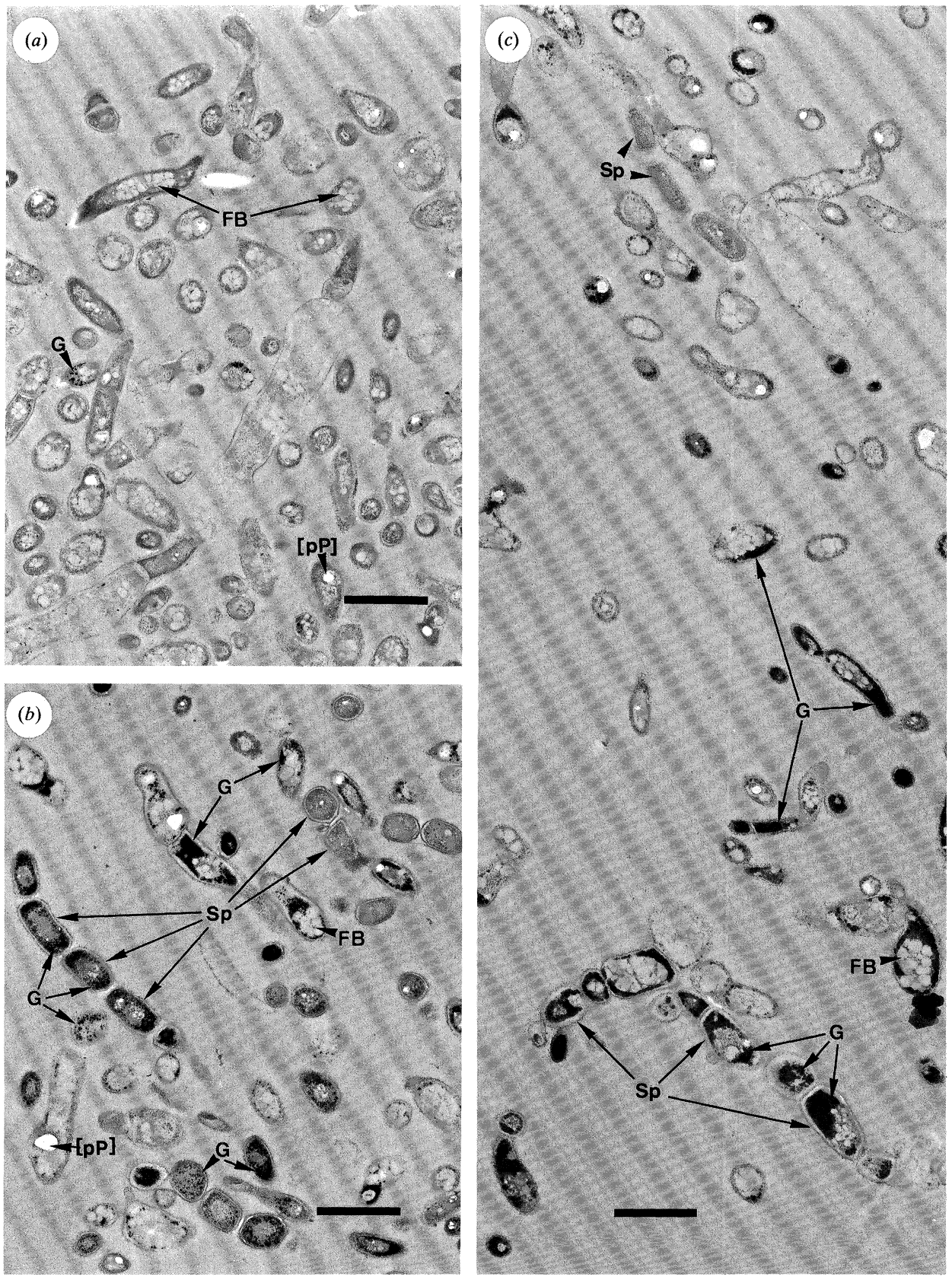

Figure 8. Medium-dependent ectopic sporulation of a *bldA* mutant induced by multiple copies of *whiG* is accompanied by glycogen deposition. The strain used was J1700 (a *bldA39* mutant) carrying pIJ4412 (a multicopy *whiG*-containing plasmid). Sections were stained with silver proteinate (*a*) 5 days, MM plus glucose (air-agar interface region); (*b*,*c*) 5 days, MM plus mannitol [(*b*): aerial mycelium; (*c*): 100 μM below the agar surface]. Key: G = glycogen granules; Sp = spore; [pP] = hole left by destruction of polyphosphate granule; FB = fatty or oily bodies. Bar markers: 2 μm.

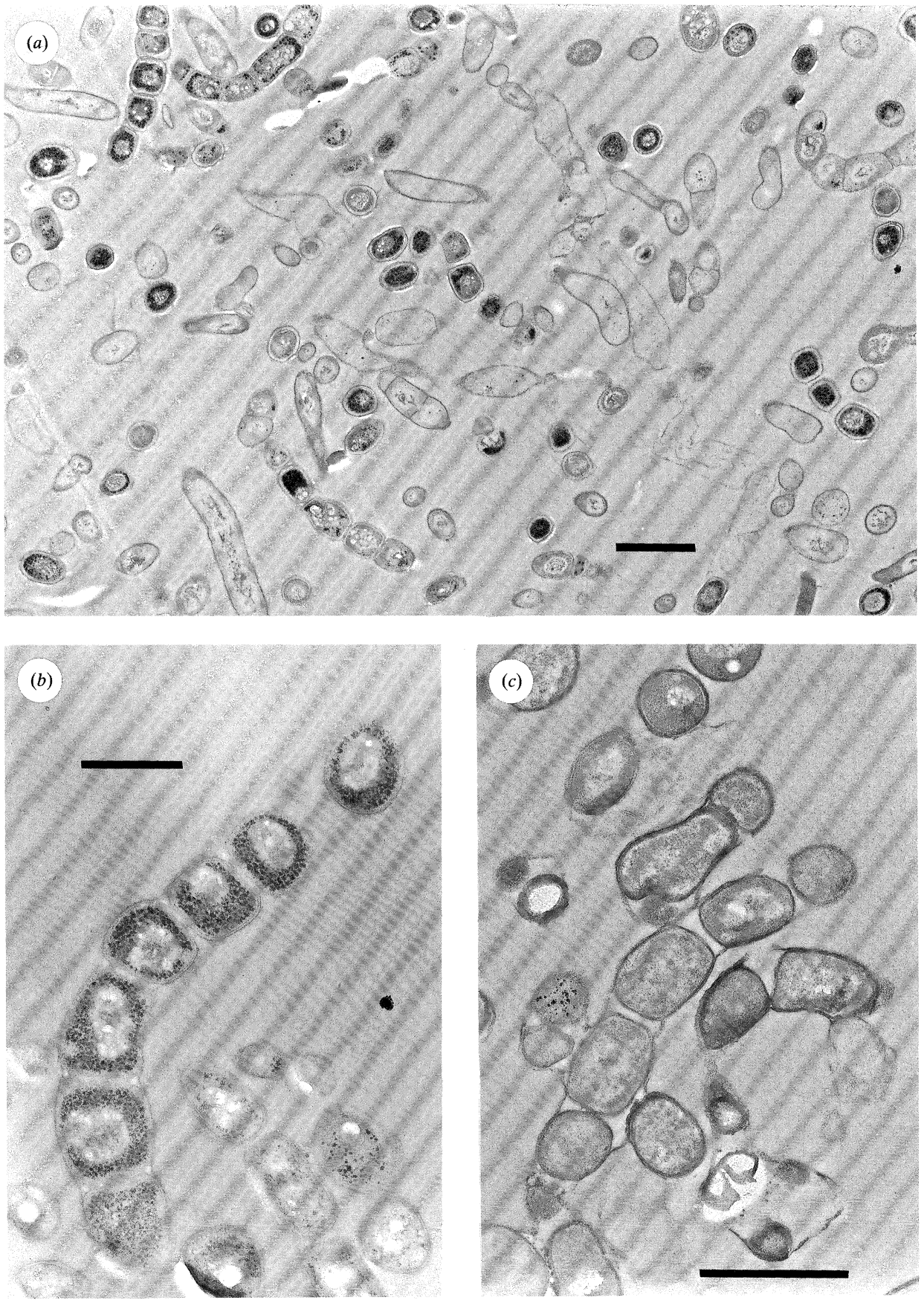

Figure 9. Effects on phase II glycogen deposition of mutations in, or extra copies of, *whiE* genes for spore pigment biosynthesis. (*a*) C107 (*whiE107*), MM plus mannitol, 4 days. Note that many well-developed spores contain very dense glycogen deposits; (*b*) C107 containing the plasmid vector pIJ922 also shows abundant glycogen deposition, MM plus mannitol, 5 days; (*c*) C107 containing extra copies of *whiE* ORFs I-VII on pIJ2156 (a pIJ922 derivative), showing almost no detectable glycogen deposits, MM plus mannitol, 4 days. Bar markers: (*a*, *c*): 2 μm; (*b*) 1 μm.

Macroscopically, and in electron microscopic analysis of thin sections, colonies of the *bldA* mutant J1700 containing pIJ4412, grown on MM plus glucose (figure 8*a*), appeared similar to control colonies of J1700 containing the pIJ702 vector portion of pIJ4412 (figure 3*a*): both showed a typical *bldA* mutant phenotype (see section 4). However, on MM plus mannitol, on which aerial mycelium formation and sporulation of *bldA* mutants such as J1700 are recovered (see section 5), J1700/pIJ4412 formed smaller, flatter colonies than J1700/pIJ702, and the substrate mycelium became almost black. Control colonies of J1700/pIJ702 developed a grey, sporulating aerial mycelium without such substrate mycelium-associated pigmentation. Both macroscopically and microscopically, the J1700/pIJ4412 colonies closely resembled colonies of the isogenic *bldA*$^+$ strain J1501/pIJ4412 grown on MM plus mannitol, exhibiting extensive sporulation on their surfaces (figure 8*b*) and within the substrate mycelium (figure 8*c*). Glycogen was abundant in developing spores in both locations (figure 8*b*, *c*), showing that this *whiG*-induced production of glycogen was epistatic to the block in phase I glycogen production in the lower parts of colonies caused by the *bldA*39 mutation.

9. INHIBITION OF AERIAL MYCELIUM-ASSOCIATED GLYCOGEN SYNTHESIS BY MULTIPLE COPIES OF *whiG*-DEPENDENT PROMOTERS

Tan & Chater (1993) reported that when either of two small *S. coelicolor* DNA fragments containing *whiG*-dependent promoters was introduced on the high copy number plasmid vector pIJ4083 into the morphologically wild-type strain J1501, colonies growing on MM plus mannitol remained white on prolonged incubation, instead of developing the grey colour associated with spore maturation. This white phenotype was associated with a reduction in sporulation, an effect believed to result from sequestering of the σ^{WhiG} form of RNA polymerase by the promoters, reducing the amount available to transcribe *whiG*-dependent sporulation genes. The association of glycogen deposition with sporulation suggested that J1501 carrying either plasmid should also be reduced in phase II glycogen deposition. Indeed, glycogen synthesis was also largely absent from aerial hyphae in these strains, and little or no glycogen was detected even in the rare developing spore chains that were found. Control cultures containing the pIJ4083 vector showed normal levels of sporulation and of phase II glycogen synthesis. (These strains were grown in the presence of thiostrepton at 7 μg ml^{-1} to maintain selection for the plasmids.)

10. GLYCOGEN ACCUMULATION IN SPORULATING AERIAL HYPHAE IS INVERSELY RELATED TO SPORE PIGMENTATION

The association of glycogen synthesis with intermediate stages in spore formation, and the absence of glycogen from mature spores, raise the question of the fate of the glycogen. One possible 'sink' would be spore pigment, which appears to contain a polyketide component (Davis & Chater 1990). Mutants (*whiE*: Chater 1972) blocked in synthesis of this polyketide might be expected to accumulate denser and more persistent deposits of glycogen. This was indeed observed with strain C107 carrying the *whiE107* mutation (figure 9*a*).

When extra copies of the cloned gene cluster carrying *whiE* ORFs I-VII were introduced (as part of plasmid pIJ2156) into *S. coelicolor*, they elicited excessive spore pigmentation (Davis & Chater 1990). If glycogen is a precursor of the *whiE*-specific pigment, then C107/pIJ2156 might be relatively depleted of glycogen in sporulating parts of its aerial hyphae. Consistent with this hypothesis, little phase II glycogen was detectable in this strain (figure 9*c*), whereas spore chains in a C107 control culture containing only the pIJ922 vector portion of pIJ2156 contained abundant glycogen (figure 9*b*). In addition, phase I glycogen deposition was also almost absent in C107/pIJ2156, but easily detected in the control strain (not shown).

11. DISCUSSION

(*a*) *Normal development and glycogen synthesis*

In *Streptomyces coelicolor* A3(2), a region of the colony at the boundary of the substrate and aerial mycelium is active in glycogen metabolism (i.e. phase I), albeit showing less dense deposition than in *S. antibioticus* (Braña *et al.* 1986). Possibly, these deposits are part of the nutrient supply for the growth of aerial hyphae. They could also provide metabolic precursors for secondary metabolism. It has also been suggested (Chater 1989) that, as a result of glycogen breakdown, osmotic pressure might increase, causing water uptake and thereby assisting the growth of hyphae into the air. Other osmotically inactive cell constituents, including triacyl glycerol (Olukoshi & Packter 1994) and proteins (Méndez *et al.* 1985; Granozzi *et al.* 1990), could also provide both nutritional and osmotic support for aerial growth (or, later, for spore development; see below). The observation that aerial mycelium could form when little or no phase I glycogen was detectable (notably during growth of several *bld* mutants on mannitol supplemented medium) shows that in these conditions glycogen is not the sole source of nutrients for aerial growth, nor the only means of providing essential turgor for this process. Although the amount of glycogen in local deposits (both phase I and phase II) is undoubtedly sufficient to cause very significant changes in cellular osmotic pressure if converted to intermediary metabolites, the extent to which these potential effects are manifested in reality will depend on the balance between the ratio of glycogen degradation and effective removal of the degradation products from the pool of osmotically active molecules (i.e. by their re-assimilation into large molecules, their export from the cell, or their volatilization as CO_2). It will be difficult to estimate such fluxes directly, so future testing of the hypothesis will probably require interference with flux by genetic manipulations.

Once formed, aerial hyphae grow until some unknown signal (involving the *whiG* gene product) causes growth to stop and spore compartments to form. Soon after ingrowth of sporulation septa is completed, abundant glycogen granules appear in the compartments. This raises the question of whether the metabolic precursors of phase II glycogen are already present in the spore compartments, or whether there is some means of transporting the precursors from the aerial hyphal stalk through many sporulation septa. Indeed, it is not excluded that transport could be extracellular, since the aerial hyphal wall is encased in a hydrophobic outer layer (Wildermuth *et al.* 1971), which might conceivably make up a type of periplasmic compartment. In addition to providing a local source of carbon and energy for sporulation, the formation of glycogen during sporulation may reduce the osmotic pressure of the spore compartments, leading to efflux of water (perhaps into the hypothetical extracellular compartment). This could balance the partial displacement of cytoplasmic volume by the developing sporulation septa. Later, the glycogen is degraded again. If there were a resultant increase in osmotic pressure any available extracellular water would be drawn back into the spore compartment, and turgor would increase again. This could assist in the generation and maintenance of the rounded shape of the spore protoplasts as the spore wall changes from its initially cylindrical shape (although the extent of any contribution of turgor pressure to changes in cell shape is not well established).

In view of the inverse correlation between the residual glycogen in sporulating compartments and the extent of pigmentation, revealed by studies of a *whiE* mutant and of a strain with additional copies of the *whiE* cluster, it seems likely that the pigment is a significant sink for carbon derived from glycogen. Other likely sinks include trehalose (Hey-Ferguson *et al.* 1973; Braña *et al.* 1986; McBride & Ensign 1990) and cell wall components. (Trehalose levels as a percentage of dry weight are considerably lower than the glycogen levels, excluding trehalose as the sole sink.) Thus, assuming the spore pigment to be associated with the spore wall, many of the osmotically active small molecules derived from glycogen would eventually be exported from the cytoplasm as pigment and cell wall precursors, taking water with them, and helping the cytoplasm to condense and acquire its relatively dehydrated, desiccation-resistant final state. This hypothesis suggests that there may be differences in cytoplasmic hydration and desiccation resistance between spores of early blocked *whiE* mutants and the wild-type. These comparisons have not been attempted, to our knowledge.

(*b*) *The studies with S. coelicolor developmental mutants and genes suggest that both phases of accumulation require the action of developmental genes*

Mycelium-associated (phase I) glycogen accumulation was seldom observed in *bldA39* mutants of *S. coelicolor* or *S. lividans*, irrespective of whether the carbon source was mannitol or glucose and hence of whether a morphologically normal aerial mycelium was present or not. This is reminiscent of the failure of *bldA* mutants of *S. coelicolor* to make any of four antibiotics produced by the wild-type, on either carbon source (Merrick 1976; Chater & Merrick 1979). Possibly, this phase of glycogen accumulation requires the action of one or more genes containing the *TTA* codon; such genes can be translated efficiently only when the wild-type *bldA* gene product, the *t*RNA for the rare leucine codon *UUA* codon, is present (Leskiw *et al.* 1991*a*, *b*). This explanation has been shown to account for actinorhodin non-production in *bldA* mutants: replacement, by site-directed mutagenesis, of the single *TTA* codon in the pathway-specific *act*IIORF4 regulatory gene allowed actinorhodin to be produced by a *bldA* mutant (Fernández-Moreno *et al.* 1991). Perhaps the mycelial phase of glycogen accumulation can be considered to be part of secondary metabolism. Most of the other representative *bld* mutants studied were also apparently deficient in phase I glycogen synthesis, in parallel with their failure to produce antibiotics. The nature and function of the products of the *bld* genes defective in these mutants remain undetermined. An exception was the *bldF* mutant, which produced abundant glycogen throughout all but the most peripheral parts of the colony. This so far genetically poorly understood mutant is unusual in other respects; it produces copious undecylprodigiosin and it seems to be unable to produce aerial mycelium or spores on any media.

The *bldA*, *bldC*, *bldD* and *bldG* mutants could be induced to undergo what seems to be normal aerial mycelium formation and sporulation by growth on MM + mannitol (this also led to ectopic sporulation in the *bldC* mutant). Sporulation of aerial hyphae was accompanied by abundant phase II glycogen synthesis in *bldA* and *bldG* mutants, showing clearly that these mutants are selectively deficient in phase I synthesis. In the *bldC* and *bldD* mutants, phase II synthesis was also detected, though only at low levels and only in a few developing spore chains; but the substantial differences in genetic background of these two mutants makes it difficult to assess the significance of these quantitative effects.

Phase II glycogen accumulation is typically very sharply demarcated in *S. coelicolor*: whenever the boundary between the non-sporulating and sporulating segments of a sectioned aerial hypha was seen, glycogen was always confined to the sporulating part. Morphologically observable initiation of sporulation septation may not be a prerequisite, because *whiB* and *whiH* (and, to a lesser extent, *whiA*) mutants which lack such septa did accumulate glycogen in the curling hyphal tips characteristic of these mutants. However, the *whiG* gene may influence sporulation-associated glycogen accumulation since there was no clear-cut association of glycogen deposition with the upper regions of aerial hyphae of *whiG* colonies (rather, phase I deposition lower in the colonies was unusually conspicuous), and phase II glycogen deposition was scarcely detectable in the rare spore chains formed by strains in which multiple copies of *whiG*-dependent

promoters appear to be sequestering the σ^{WhiG} form of RNA polymerase. A direct or indirect role for *whiG* in phase II glycogen synthesis was more strongly indicated by the observation that *whiG*-induced ectopic sporulation was always accompanied by glycogen deposition, even in a *bldA* mutant that otherwise showed almost no glycogen synthesis in the lower parts of colonies.

The sporulation-associated (phase II) glycogen accumulation observed in *bldA* mutant colonies growing and sporulating on MM plus mannitol indicates that the genes for this phase of accumulation contain no *TTA* codons, since the dependence on *bldA* of *TTA*-containing genes is not alleviated by growth on mannitol (Leskiw *et al.* 1991*a*). The question of whether each phase of accumulation involves a separate set of structural genes, or whether the same set of genes (but with two alternative modes of regulation) is used for both phases, will be best studied with cloned genes for glycogen synthesis, some candidates for which have now been cloned from streptomycetes (C.J. Bruton, personal communication; M.C. Martin & C. Hardisson, personal communication; J. Kormanec, personal communication). It will be of equal interest to investigate the activity of glycogen degradative enzymes during development.

(*c*) *Storage metabolism and morphogenesis during microbial sporulation*

The localization of glycogen deposits in *Streptomyces* colonies, and the evidence for regulation by genes involved in morphological differentiation, has led us to propose [see section (a), above] that glycogen metabolism plays a significant role in morphogenesis by contributing to changes in turgor, and that other storage compounds may also be contributors. Such a role may not be confined to streptomycetes and their near relatives. Glycogen and/or other storage compounds accumulate in the early stages of sporulation in various endospore-forming bacteria and are then degraded as sporulation proceeds (Tinelli 1955; Strasdine 1972; Slock & Stahly 1974; Mackay & Morris 1975; Bergère *et al.* 1985; Lowe *et al.* 1989). Where studied, glycogen biosynthetic genes in most of these organisms appear to have promoters recognized by an RNA polymerase form containing σ^{H} (Kiel *et al.* 1991, 1992, 1993), a transcription factor important in activating genes for sporulation and competence for transformation (Dubnau 1989; Moran 1989). In *Bacillus subtilis*, the glycogen biosynthetic genes are apparently expressed from a different class of sporulation-specific promoter, recognized by the spore mother cell-specific sigma factor σ^{E} (Kiel *et al.* 1994). We suggest that differences in osmotic pressure between the forespore and mother cell compartments could result from controlled metabolism of storage compounds, and might assist, or even be responsible for, the crucial – but mechanistically obscure – engulfment of the former by the latter. Glycogen synthesis and degradation also take place in *Saccharomyces cerevisiae* cells at the time of ascospore formation, the morphological description of which implies localized redistribution of subcellular volumes (Hopper *et al.* 1974; Kane & Rother 1974; Colonna & Magee 1978); and at least one enzyme for glycogen degradation in yeast is under sporulation-specific control (Smith *et al.* 1988). Thus, although there is obvious potential value of glycogen as a store of carbon and energy for sporulation processes, its role as an indirect morphogenetic agent may deserve investigation.

There is some general support for the notion that glycogen metabolism during sporulation could well effect significant turgor changes. Strong circumstantial evidence suggests an involvement of glycogen synthesis and degradation in the light-dependent control of buoyancy via turgor pressure in the cyanobacteria *Spirulina platensis* (Warr *et al.* 1985) and *Microcystis aeruginosa* (Kromkamp & Mur 1984). Among eukaryotes, glycogen synthesis is stimulated in liver cells as a response to swelling (Meijer et al. 1992); in some plants the starch levels in stomatal guard cells fall as stomata open, in association with increased turgor, and increase as stomata close, in association with decreased turgor (Willmer 1981); and in the classical case of wrinkled (morphologically abnormal) peas (Mendel 1865), the inactivation of starch branching enzyme by transposon insertion at the R locus (Battacharrya *et al.* 1990) causes increased osmotic potential and water content in developing embryonic tissues (Wang *et al.* 1987). It would almost be surprising if in microbes the morphogenetic potential implicit in these storage macromolecules had remained unrecognized by the forces of evolution.

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REFERENCES

Battacharrya, M.K., Smith, A.M., Ellis, T.H.N., Hedley, C.L. & Martin, C. 1990 The wrinkled seed character of pea described by Mendel is caused by a transposon-like insertion in a gene encoding starch-branching enzyme. *Cell* **60**, 115–122.

Bergère, J.L., Rousseau, M. & Mercier, C. 1975 Polyoside intracellulaire impliqué dans la sporulation de *Clostridium butyricum*. *Ann. Microbiol.* **126A**, 295–314.

Braña, A.F., Manzanal, M.B. & Hardisson, C. 1980 Occurrence of polysaccharide granules in sporulating hyphae of *Streptomyces viridochromogenes*. *J. Bact.* **144**, 1139–1142.

Braña, A.F., Manzanal, M.B. & Hardisson, C. 1982 Characterization of intracellular polysaccharides of *Streptomyces*. *Can. J. Microbiol.* **28**, 1320–1323.

Braña, A.F., Méndez, C., Díaz, L.A., Manzanal, M.B. & Hardisson, C. 1986 Glycogen and trehalose accumulation during colony development in *Streptomyces antibioticus*. *J. Gen. Microbiol.* **132**, 1319–1326.

Champness, W.C. 1988 New loci required for *Streptomyces coelicolor* morphological and physiological differentiation. *J. Bact.* **170**, 1168–1174.

Champness, W.C. & Chater, K.F. 1994 The regulation and integration of antibiotic production and morphological

differentiation in *Streptomyces* spp. In *Regulation of bacterial differentiation* (ed. P.J. Piggot, C.P. Moran, & P. Youngman), pp. 61–93. Washington D.C.: American Society for Microbiology.

Chater, K.F. 1972 A morphological and genetic mapping study of white colony mutants of *Streptomyces coelicolor*. *J. Gen. Microbiol.* **72**, 9–28.

Chater, K.F. 1989 Multilevel regulation of *Streptomyces* differentiation. *TIG.* **5**, 372–376.

Chater, K.F. 1993 Genetics of differentiation in *Streptomyces*. *A. Rev. Microbiol.* **47**, 685–713.

Chater, K.F., Bruton, C.J., King, A.A. & Suárez, J.E. 1982 The expression of *Streptomyces* and *Escherichia coli* drug resistance determinants cloned into the *Streptomyces* phage øC31. *Gene* **19**, 21–23.

Chater, K.F., Bruton, C.J., Plaskitt, K.A., Buttner, M.J., Méndez, C. & Helmann, J. 1989 The development fate of *S. coelicolor* hyphae depends crucially on a gene product homologous with the motility sigma factor of *B. subtilis*. *Cell* **59**, 133–143.

Chater, K.F. & Merrick, M.J. 1979 *Streptomyces*. In *Developmental biology of prokaryotes* (ed. J.H. Parish), pp. 93–114. Oxford: Blackwell.

Clayton, T.M. & Bibb, M.J. 1990 *Streptomyces* promoter-probe plasmids that utilise the *xylE* gene of *Pseudomonas putida*. *Nucl. Acids Res.* **18**, 1077.

Cohen, P. 1988 Protein phosphorylation and hormone action. *Proc. R. Soc. Lond.* B **234**, 115–144.

Colonna, W.J. & Magee, P.T. 1978 Glycogenolytic enzymes in sporulating yeast. *J. Bact.* **134**, 844–853.

Davis, N.K. & Chater, K.F. 1990 Spore colour in *Streptomyces coelicolor* A3(2) involves the developmentally regulated synthesis of a compound biosynthetically related to polyketide antibiotics. *Molec. Microbiol.* **4**, 1679–1691.

Davis, N.K. & Chater, K.F. 1992 The *Streptomyces coelicolor whiB* gene encodes a small transcription factor-like protein dispensable for growth but essential for sporulation. *Molec. gen. Genet.* **232**, 351–358.

Dubnau, D. 1989 The competence regulon of *Bacillus subtilis*. In *Regulation of prokaryotic development* (ed. I. Smith, R. Slepecky, & P. Setlow), pp. 147–166. Washington D.C.: American Society for Microbiology.

Eckhardt, T. & Smith, R. 1987 Use of a secreted β-galactosidase to study expression and secretion in *Streptomyces*. In *Genetics of industrial microorganisms, part B* (ed. M. Alačević, D. Hranueli & Z. Toman), pp. 17–27. Zagreb: Pliva.

Fernández-Moreno, M.A., Caballero, J.L., Hopwood, D.A. & Malpartida, F. 1991 The *act* cluster contains regulatory and antibiotic export genes, direct targets for translational control by the *bldA* tRNA gene of *Streptomyces*. *Cell* **66**, 769–780.

Glauert, A.M. & Brieger, E.M. 1955 The electron-dense bodies of *Mycobacterium* *J. Gen. Microbiol.* **13**, 310–317.

Granozzi, C., Billeta, R., Passantino, R., Sollazzo, M., & Puglia, A.M. 1990. A breakdown in macromolecular synthesis preceding differentiation in *Streptomyces coelicolor* A3(2). *J. gen. Microbiol.* **135**, 713–718.

Hey-Ferguson, A., Mitchell, M. & Elbein, A.D. 1973 Trehalose metabolism in germinating spores of *Streptomyces hygroscopicus*. *J. Bact.* **116**, 1084–1085.

Hopper, A.K., Magee, P.T., Welch, S.K., Friedman, M. & Hall, B.D. 1974 Macromolecular synthesis and breakdown in relation to sporulation and meiosis in yeast. *J. Bact.* **119**, 619–629.

Hopwood, D.A., Bibb, M.J., Chater, K.F., Kieser, T., Bruton, C.J., Kieser, H.M., Lydiate, D.J., Smith, C.P., Ward, J.M. & Schrempf, H. 1985 *Genetic manipulation of Streptomyces. A laboratory manual.* Norwich: John Innes Foundation.

Hopwood, D.A. & Glauert, A.M. 1960 The fine structure of *Streptomyces coelicolor*. II. The nuclear material. *J. Biophys. Biochem. Cytol.* **8**, 267–278.

Hopwood, D.A., Wildermuth, H. & Palmer, H.M. 1970 Mutants of *Streptomyces coelicolor* defective in sporulation. *J. gen. Microbiol.* **61**, 397–408.

Jenner, C.F. 1982 Storage of starch.In *Encyclopaedia of plant physiology, New Series, Vol. 13A, Plant Carbohydrates I: Intracellular Carbohydrates* (ed. F.A. Loemus & W. Tanner), pp. 700–747. Berlin: Springer–Verlag.

Kane, S.M. & Roth, R. 1974 Carbohydrate metabolism during microspore development in yeast. *J. Bact.* **118**, 8–14.

Kiel, J.A.K.W., Boels, J.M., Beldman, G. & Venema, G. 1991 Molecular cloning and nucleotide sequence of the glycogen branching enzyme gene (*glgB*) from *Bacillus stearothermophilus* and expression in *Escherichia coli* and *Bacillus subtilis*. *Mol. gen. Genet.* **230**, 136–144.

Kiel, J.A.K.W., Boels, J.M., Beldman, G. & Venema, G. 1992 The *glgB* gene from the thermophile *Bacillus caldolyticus* encodes a thermolabile branching enzyme. *DNA Sequ. J. DNA Sequ. & Map.* **3**, 221–232.

Kiel, J.A.K.W., Boels, J.M., Beldman, G. & Venema, G. 1994 Glycogen in *Bacillus subtilis*: molecular characterization of an operon encoding enzymes involved in glycogen biosynthesis and degradation. *Molec. Microbiol.* **11**, 203–218.

Kiel, J.A.K.W., Boels, J.M., Ten Berge, A.M. & Venema, G. 1993 Two putative insertion sequences flank a truncated glycogen branching enzyme gene in the thermophile *Bacillus stearothermophilus* CU21. *DNA Sequ. J. DNA Sequ. & Map.* **4**, 1–9.

Kromkamp, J.C. & Mur, L.R. 1984 Buoyant density changes in the cyanobacterium *Microcystis aeruginosa* due to changes in the cellular carbohydrate content. *FEMS Microbiol. Lett.* **25**, 105–109.

Lawlor, E.J., Baylis, H.A. & Chater, K.F. 1987 Pleiotropic morphological and antibiotic deficiencies result from mutations in a gene encoding a tRNA-like product in *Streptomyces coelicolor* A3(2). *Genes Dev.* **1**, 1305–1310.

Leskiw, B.K., Bibb, M.J. & Chater, K.F. 1991*a* The use of a rare codon specifically during development? *Molec. Microbiol.* **5**, 2861–2867.

Leskiw, B.K., Lawlor, E.J., Fernández-Abalos, J.M. & Chater, K.F. 1991*b* TTA codons in some genes prevent their expression in a class of developmental, antibiotic-negative *Streptomyces* mutants. *Proc. natn. Acad. Sci. U.S.A.* **88**, 2461–2465.

Lowe, S.E., Pankratz, H.S. & Zeikus, J.G. 1989 Influence of pH extremes on sporulation and ultrastructure of *Sarcina ventriculi*. *J. Bact.* **171**, 3775–3781.

McBride, M.J. & Ensign, J.C. 1990 Regulation of trehalose metabolism by *Streptomyces griseus* spores. *J. Bact.* **172**, 3637–3643.

McVittie, A.M. 1974 Ultrastructural studies on sporulation in wild-type and white colony mutants of *Streptomyces coelicolor*. *J. gen. Microbiol.* **81**, 291–302.

Meijer, A.J, Baquet, A., Gustafson, L., van Woerkom, G.M. & Hue, L. 1992 Mechanism of activation of liver glycogen synthase by swelling. *J. biol. Chem.* **267**, 5823–5828.

Mendel, G. 1865 Versüche über Pflanzen-Hybriden. Verhandlungen des naturforschenden Vereines in Brünn IV.

Méndez, C., Braña, A.F., Manzanal, M.B. & Hardisson, C. 1985. Role of substrate mycelium in colony development in *Streptomyces*. *Can. J. Microbiol.* **31**, 446–450.

Méndez, C. & Chater, K.F. 1987 Cloning of *whiG*, a gene

critical for sporulation of *Streptomyces coelicolor* A3(2). *J. Bact.* **169**, 5715–5720.

Merrick, M.J. 1976 A morphological and genetic mapping study of bald colony mutants of *Streptomyces coelicolor*. *J. gen. Microbiol.* **96**, 299–315.

Moran, C.P. 1989 Sigma factors and the regulation of transcription. In *Regulation of prokaryotic development* (ed. I. Smith, R. Slepecky, & P. Setlow), pp. 167–184. Washington D.C.: American Society for Microbiology.

Olukoshi, E.R. 1993 Metabolism of triacylglycerol in *Streptomyces*. Ph.D. Thesis, University of Leeds.

Olukoshi, E.R. & Packter, N.M. 1994 Importance of stored triacylglycerols in *Streptomyces*: possible carbon source for antibiotics. *Microbiology* **140**, 931–943.

Passantino, R., Puglia, A.M. & Chater, K.F. 1991 Additional copies of the *act*II regulatory gene induce actinorhodin production in pleiotropic *bld* mutants of *Streptomyces coelicolor* A3(2). *J. gen. Microbiol.* **137**, 2059–2064.

Preiss, J. & Romeo, T. 1989 Physiology, biochemistry and genetics of bacterial glycogen synthesis. *Adv. microb. Physiol.* **30**, 183–238.

Ramade, N. & Vining, L.C. 1993 Accumulation of intracellular carbon reserves in relation to chloramphenicol biosynthesis by *Streptomyces venezuelae*. *Can. J. Microbiol.* **39**, 377–383.

Robertson, J.G., Lyttleton, P., Williamson, K.I. & Batt, R.D. 1975 Effect of fixation procedures on the electron density of polysaccharide granules in *Nocardia corallina*. *J. Ultrastruct. Res.* **50**, 321–332.

Slock, J.A. & Stahly, D.P. 1974 Polysaccharide that may serve as a carbon and energy storage compound for sporulation in *Bacillus cereus*. *J. Bact.* **120**, 399–406.

Smith, L.M., Robbins, L.G., Kennedy, A. & Magee, P.T. 1988 Identification and characterization of mutations affecting sporulation in *Saccharomyces cerevisiae*. *Genetics* **120**, 899–907.

Strasdine, G.A. 1972 The role of intracellular glucan in endogenous fermentation and spore maturation in *Clostridium botulinum* type E. *Can. J. Microbiol.* **18**, 211–217.

Tan, H. & Chater, K.F. 1992 Two developmentally controlled promoters of *Streptomyces coelicolor* A3(2) that resemble the major class of motility-related promoters in other bacteria. *J. Bact.* **175**, 933–940.

Thiéry, J.P. 1967 Mise en évidence de polysaccharides sur coupe fines en microscopie électronique. *J Microsc., Paris* **6**, 987–1018.

Tinelli, R. 1955 Etude de la biochimie de la sporulation chez *Bacillus megaterium*. I. Composition des spores obtenu par carence des différents substrats carbones. *Annls Inst. Pasteur, Paris* **88**, 212–226.

Wang, T.L., Smith, C.M., Cook, S.K., Ambrose, M.J. & Hedley, C.L. 1987 An analysis of seed development in *Pisum sativum*. III. The relationship between the *r* locus, the water content and the osmotic potential of seed tissues *in vivo* and *in vitro*. *Ann. Bot.* **59**, 73–80.

Warr, S.C.R., Reed, R.H., Chudek, J.A., Foster, R. & Stewart, W.D.P. 1985 Osmotic adjustment in *Spirulina platensis*. *Planta* **163**, 424–429.

Willmer, C.M. 1981 Guard cell metabolism. In *Stomatal physiology* (ed. P.G. Jarvis and T.A. Mansfield), pp. 87–102. Cambridge: Cambridge University Press.

Wildermuth, H. 1970 Development and organisation of the aerial mycelium in *Streptomyces coelicolor*. *J. gen. Microbiol.* **60**, 43–50.

Wildermuth, H. & Hopwood, D.A. 1970 Septation during sporulation in *Streptomyces coelicolor*. *J. gen. Microbiol.* **60**, 51–59.

Wildermuth, H., Wehrli, E. & Horne, R.W. 1971 The surface structure of spores and aerial mycelium in *Streptomyces coelicolor*. *J. Ultrastruct. Res.* **35**, 168–180.

Wright, F. & Bibb, M.J. 1992 Codon usage in the G + C-rich *Streptomyces* genome. *Gene* **113**, 55–65.

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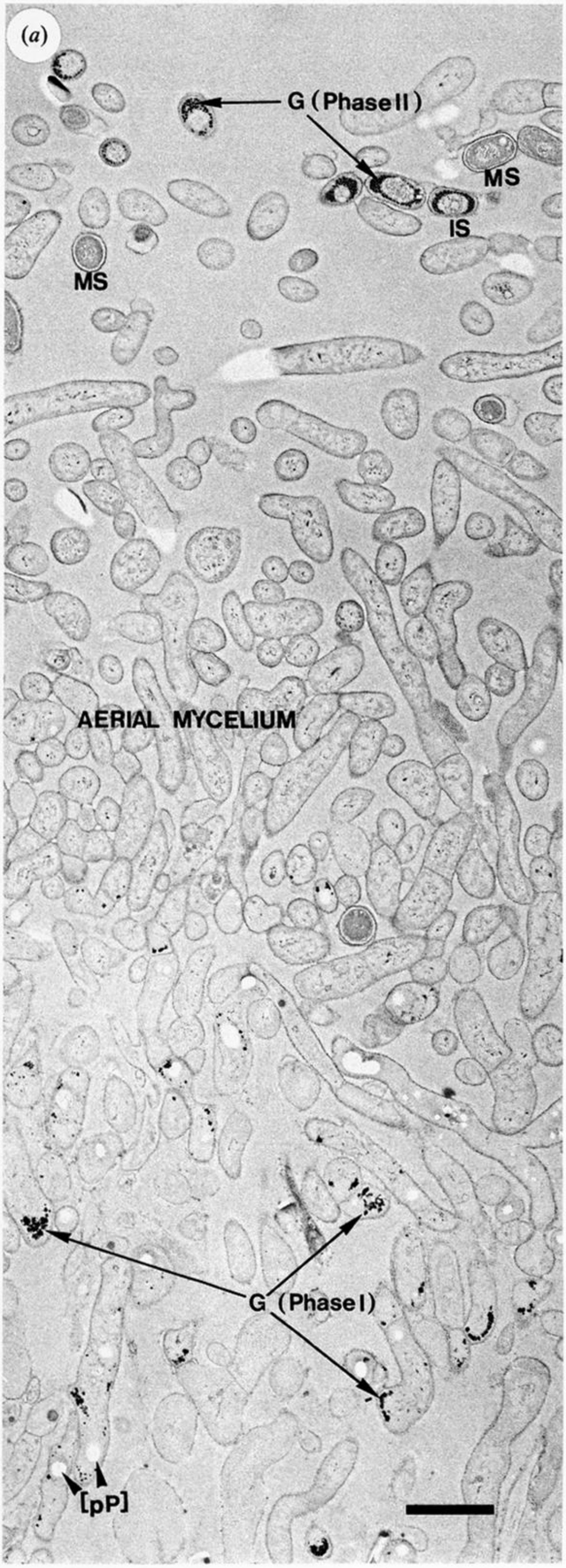
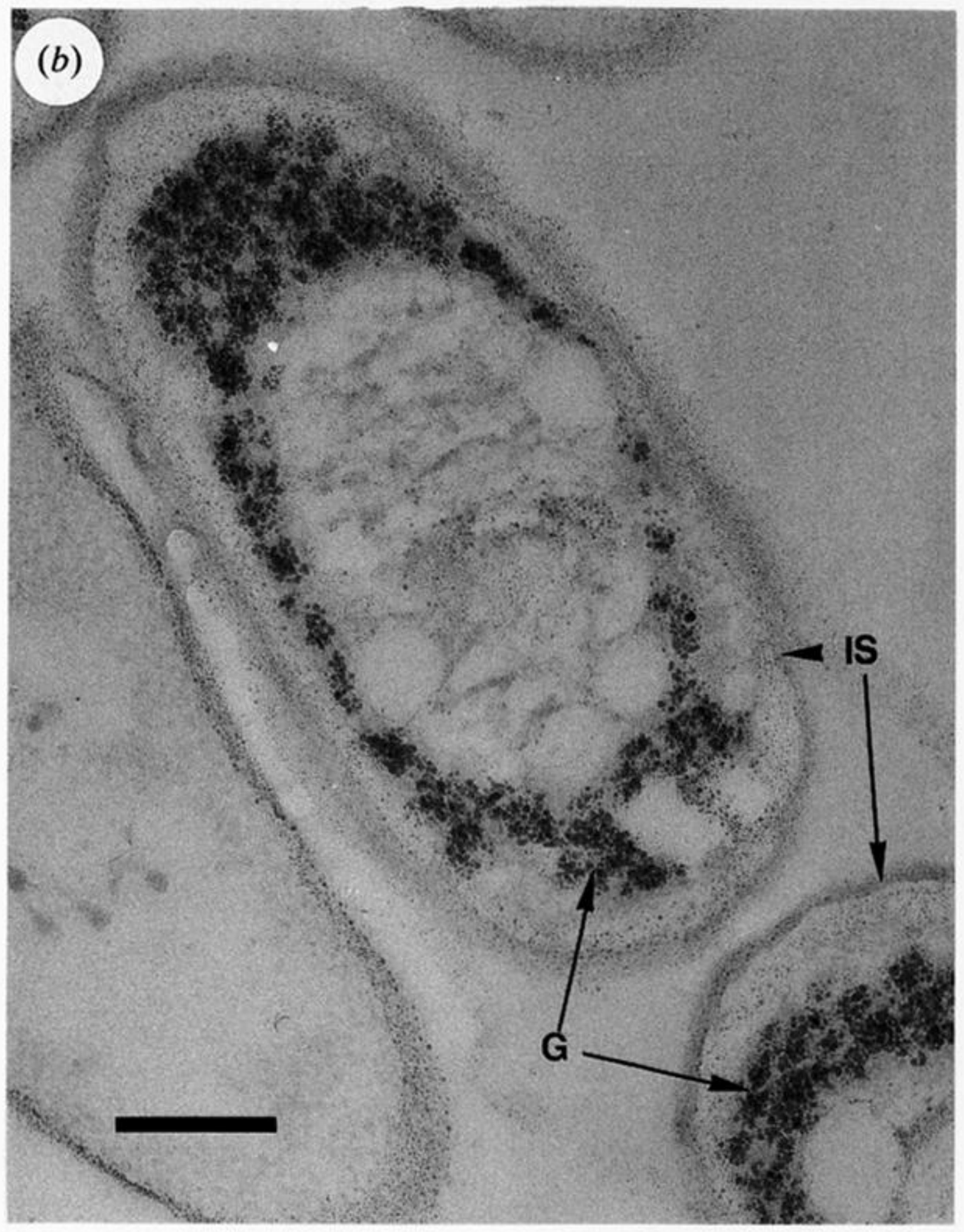




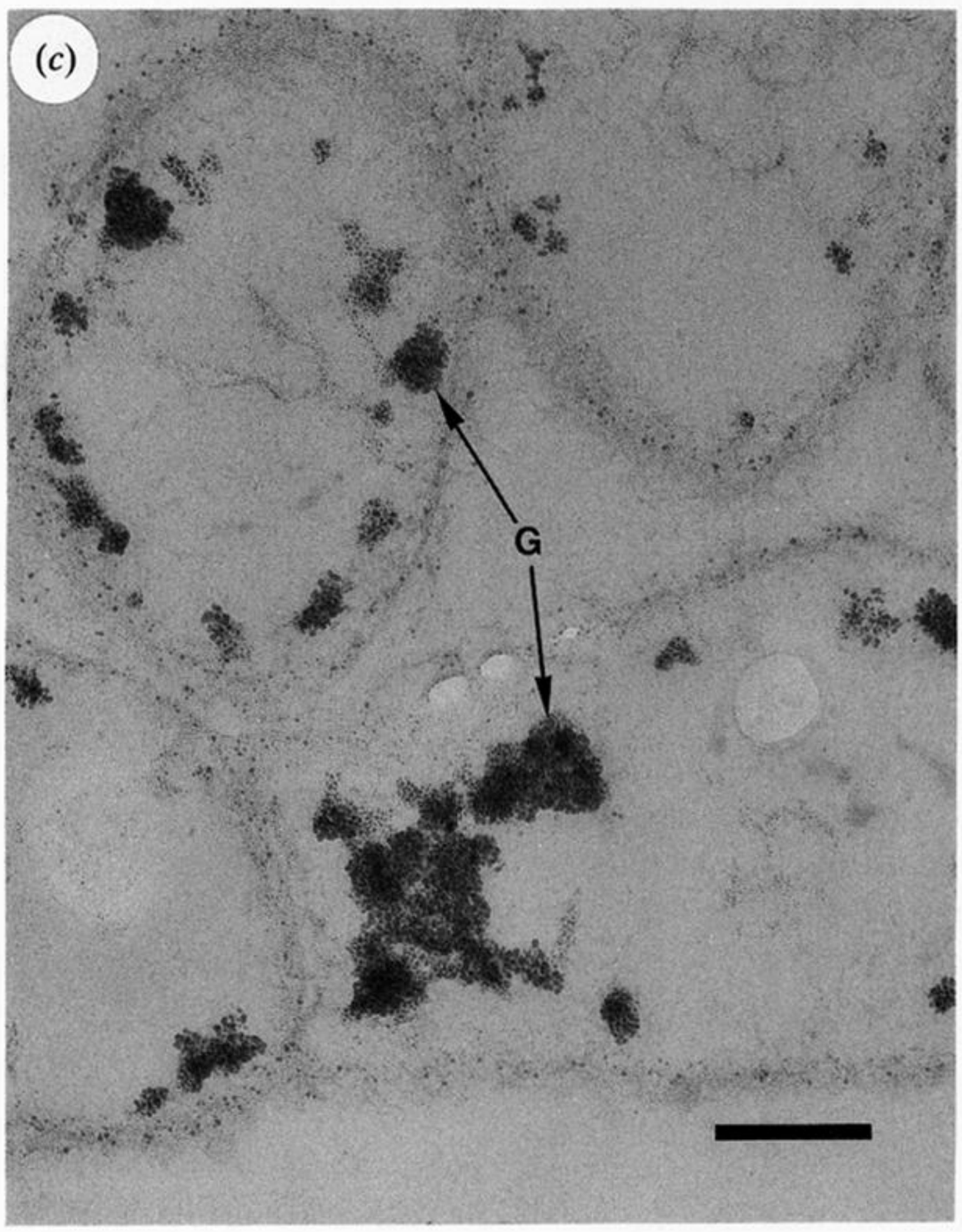


Figure 1. Two phases of glycogen synthesis in *S. coelicolor* A3(2). (*a*) Section extending from the upper surface to the centre of a colony. At a low abundance, glycogen granules are visible in the lower part of the mycelium (phase I). Glycogen is absent from aerial hyphae, but abundant in immature spore chains (phase II). As spores mature and develop thick walls, glycogen disappears. (*b*) Phase II, higher magnification. (*c*) Phase I, higher magnification. All sections were of an *S. coelicolor* A3(2) colony grown for 5 days directly on MM+mannitol. Key: G = glycogen; IS = immature spore; MS = mature spore; [pP] = hole left by destruction of polyphosphate granules. Bar markers: (*a*) 2 μm; (*b*, *c*) 200 nm.

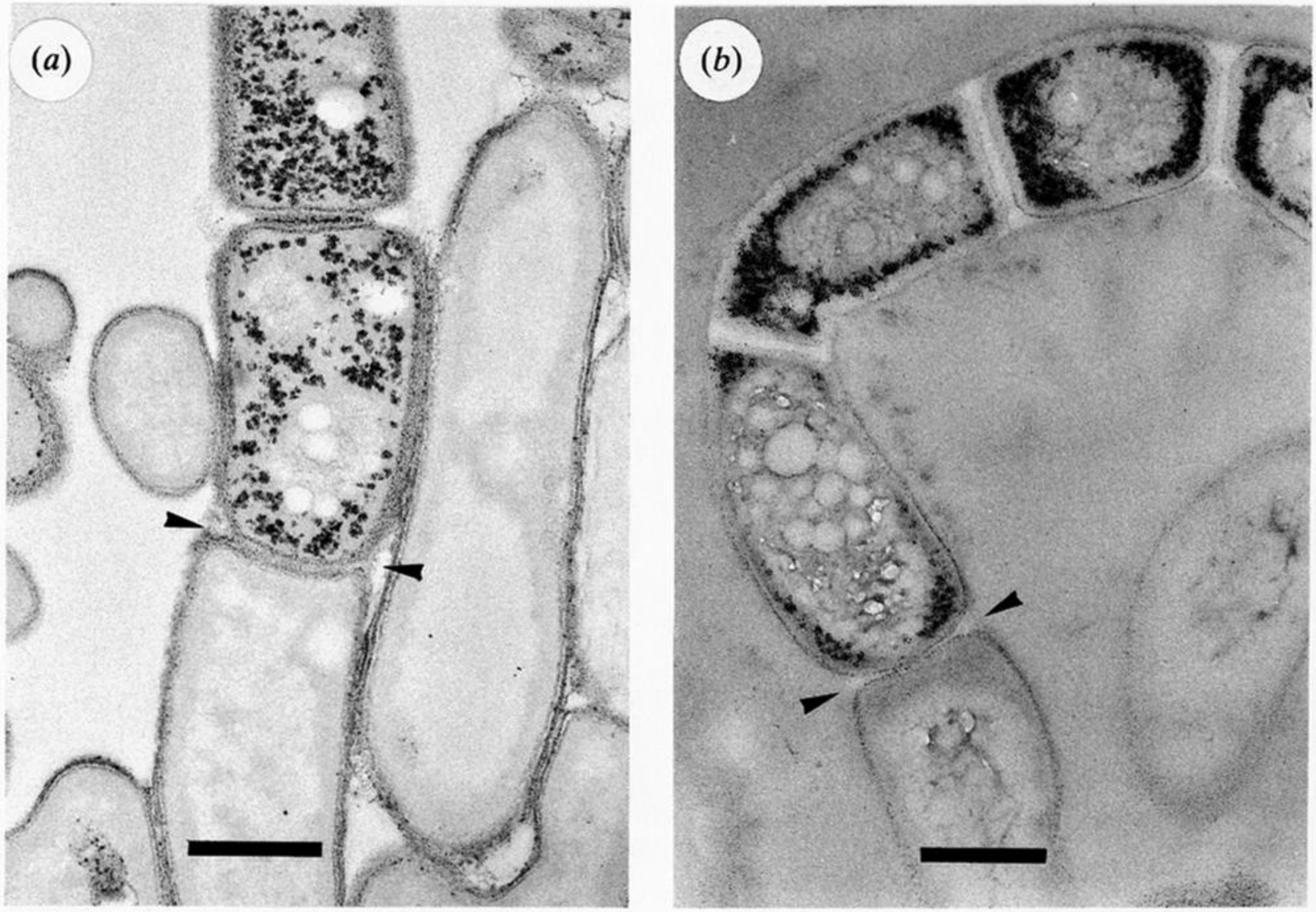
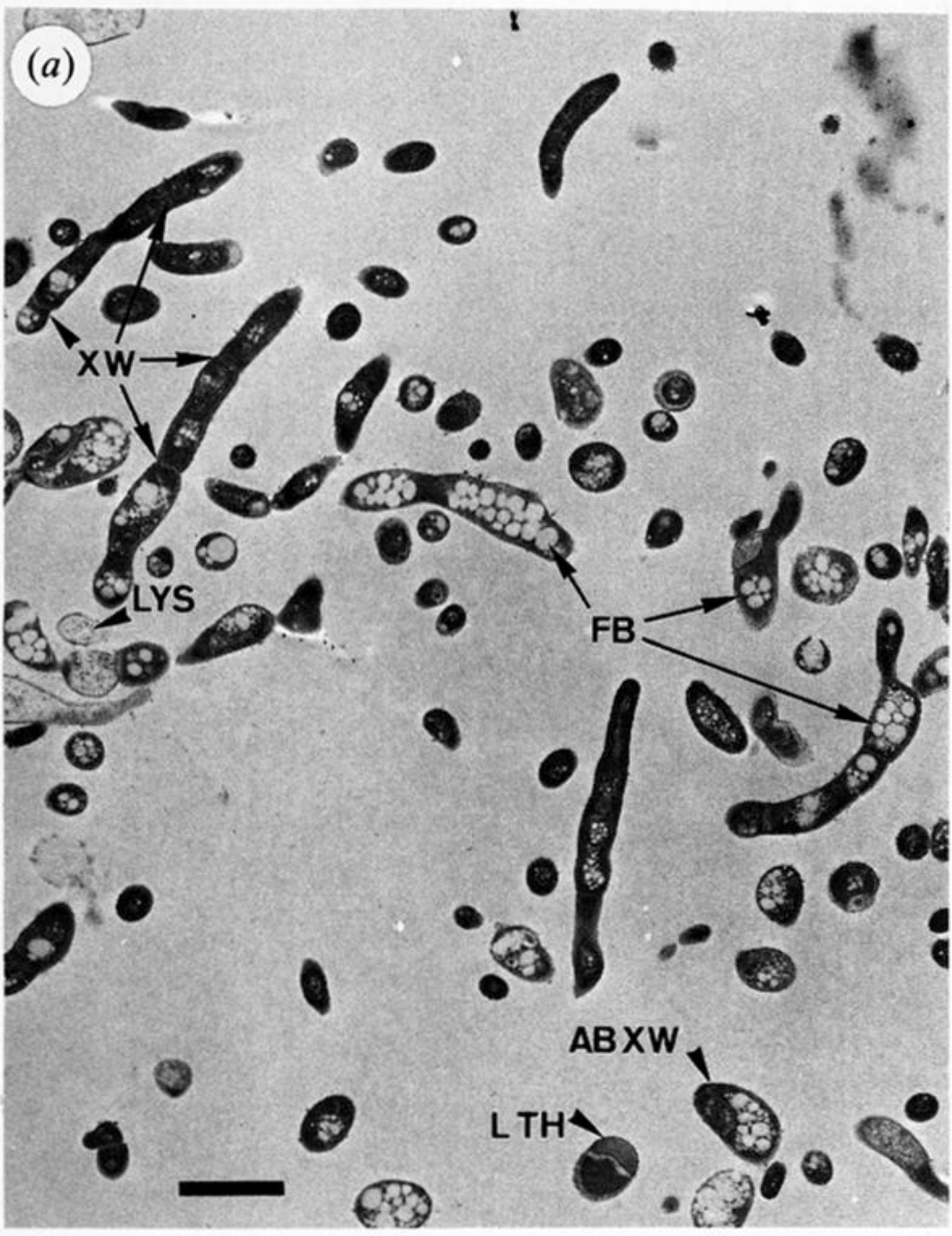
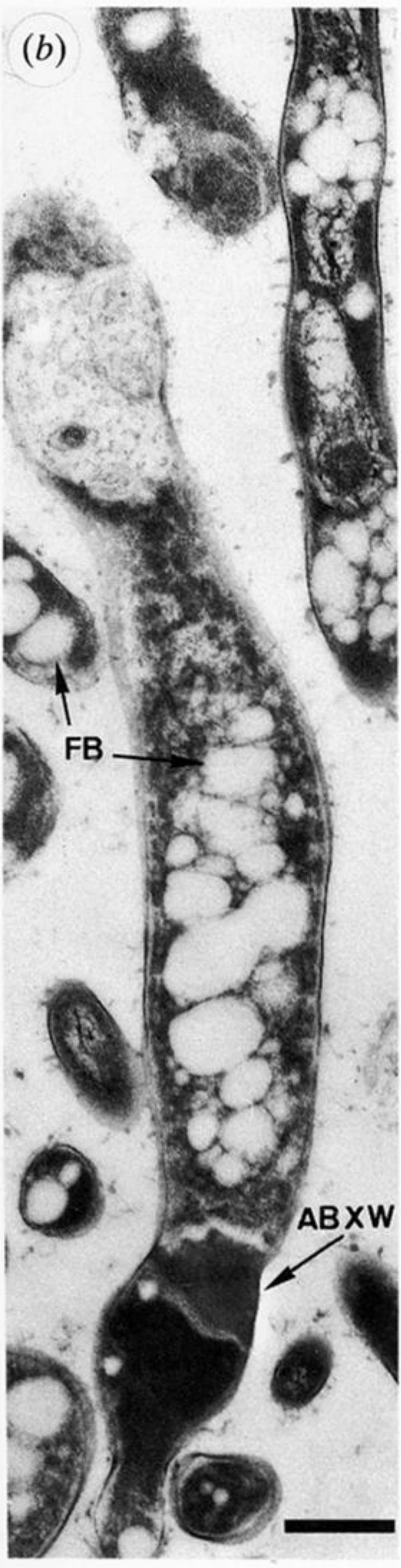


Figure 2. A sharp discontinuity of glycogen synthesis at the cross-walls (arrowed) separating pre-spore chains from their supporting aerial hyphae. Note also that the two faces of the arrowed cross-walls differ in the way in which they join the lateral wall (see text). (*a*) Strain J1501/pIJ486, MM plus mannitol, 4 days. (*b*) Strain A3(2), cellophane, MM plus 2% glucose, 5.75 days. Bar markers, 500 nm.





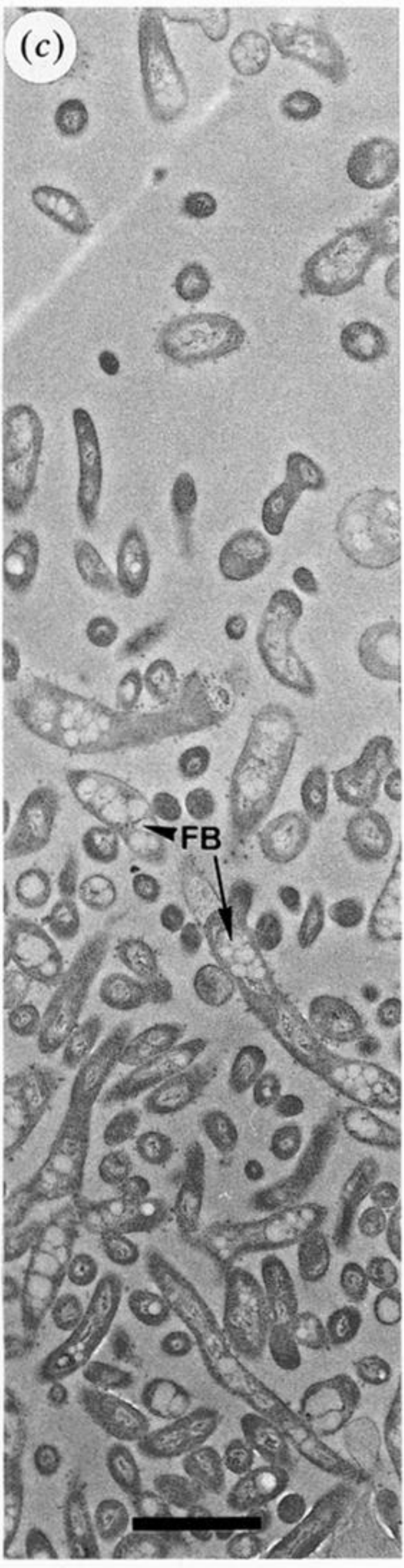


Figure 3. During growth on glucose, a *bldA* mutant makes no spores and accumulates little glycogen. (*a*) Uppermost cells of colony of J1700 (in this case containing the vector pIJ702), 5 days, uranyl acetate stain. (*b*) Higher magnification of part of (*a*). (*c*) J1700, uppermost cells, 7 days, silver proteinate stain (note absence of darkly stained glycogen granules). Key: ABXW = aberrant cross-wall; LTH = irregular lateral thickening of cell wall; LYS = lysed cell; XW = cross-wall; FB = fatty or oily bodies. Bar markers: (*a–c*) 2 μm; (*b*) 500 nm.

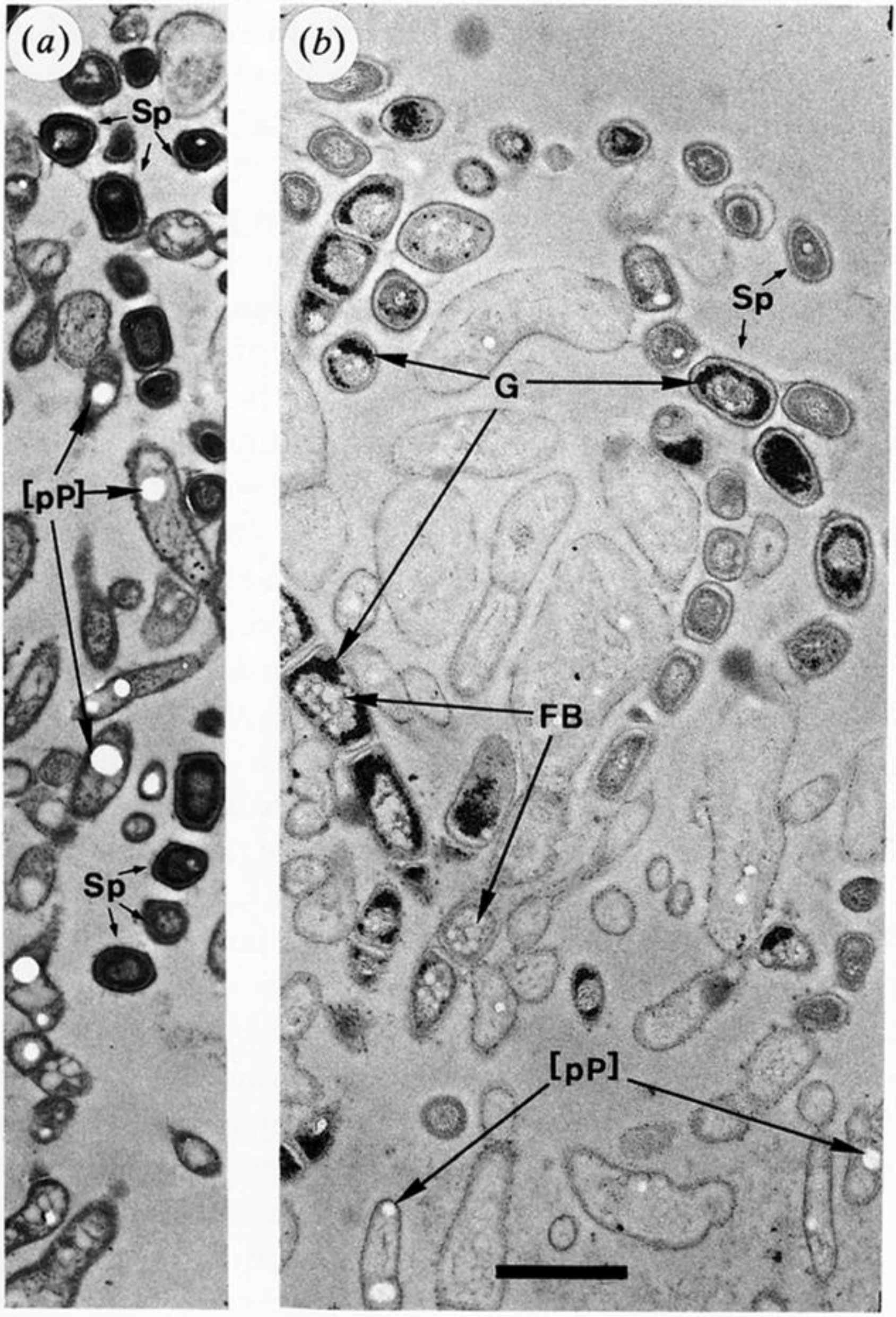
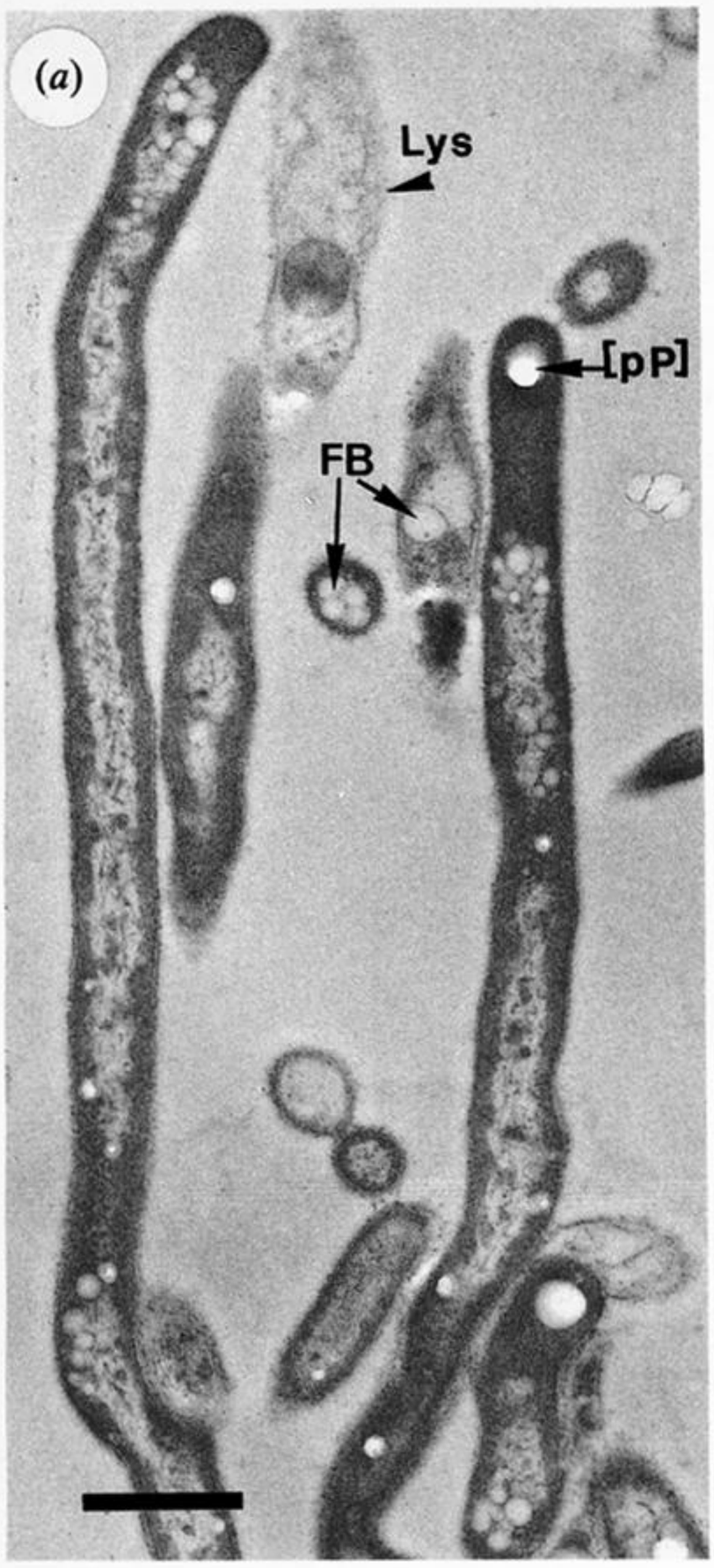
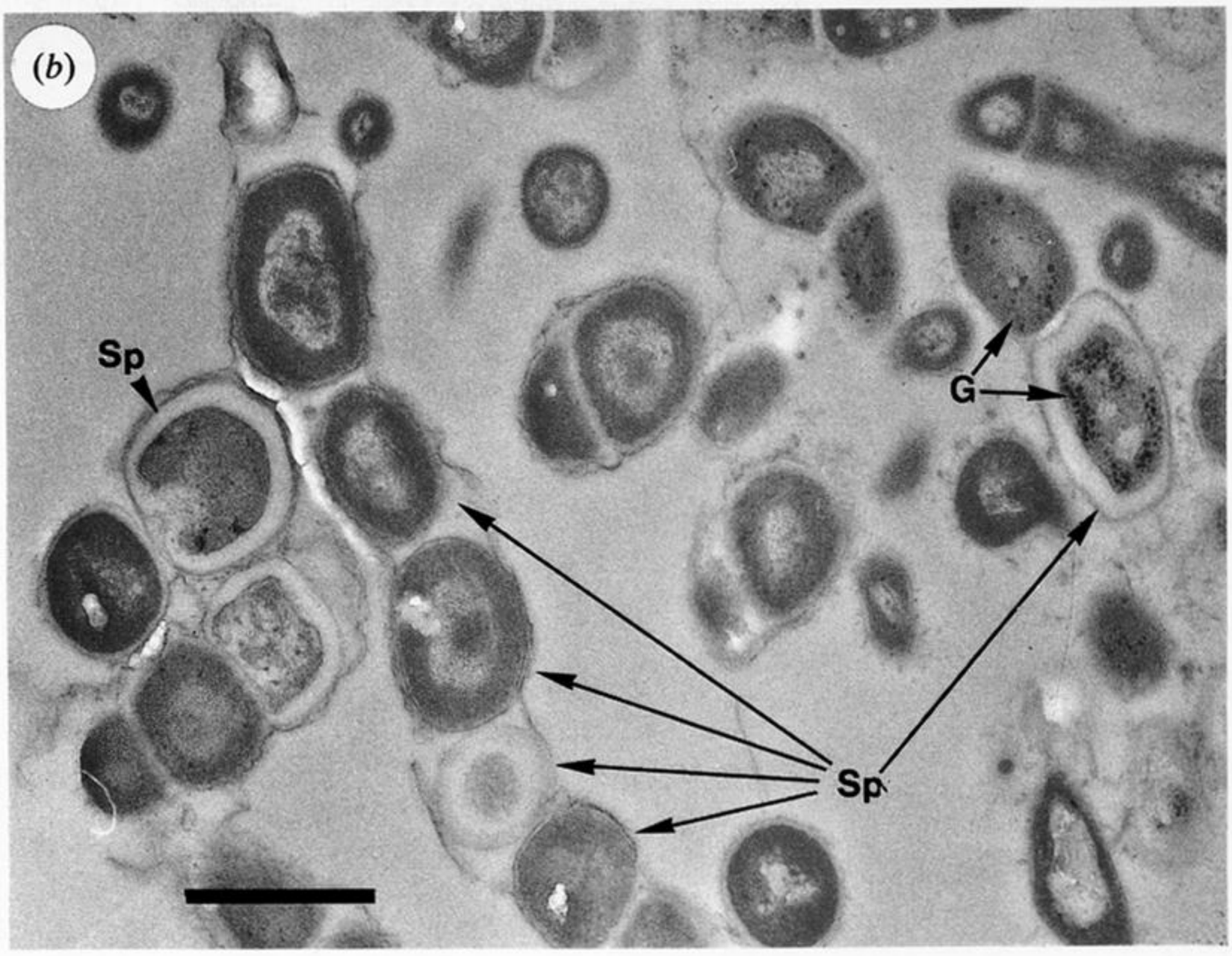
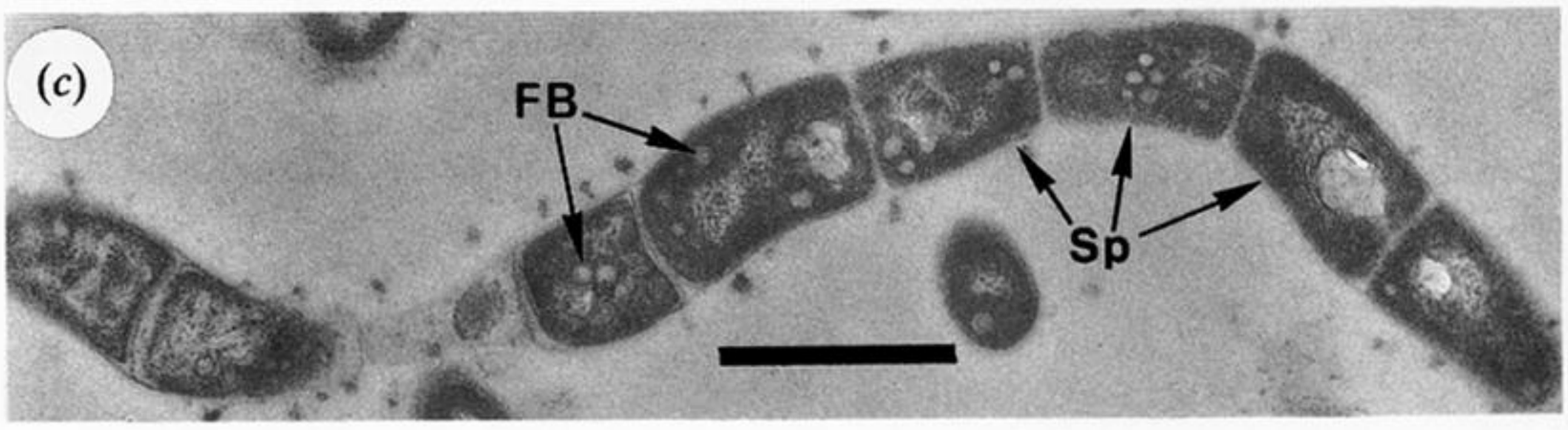
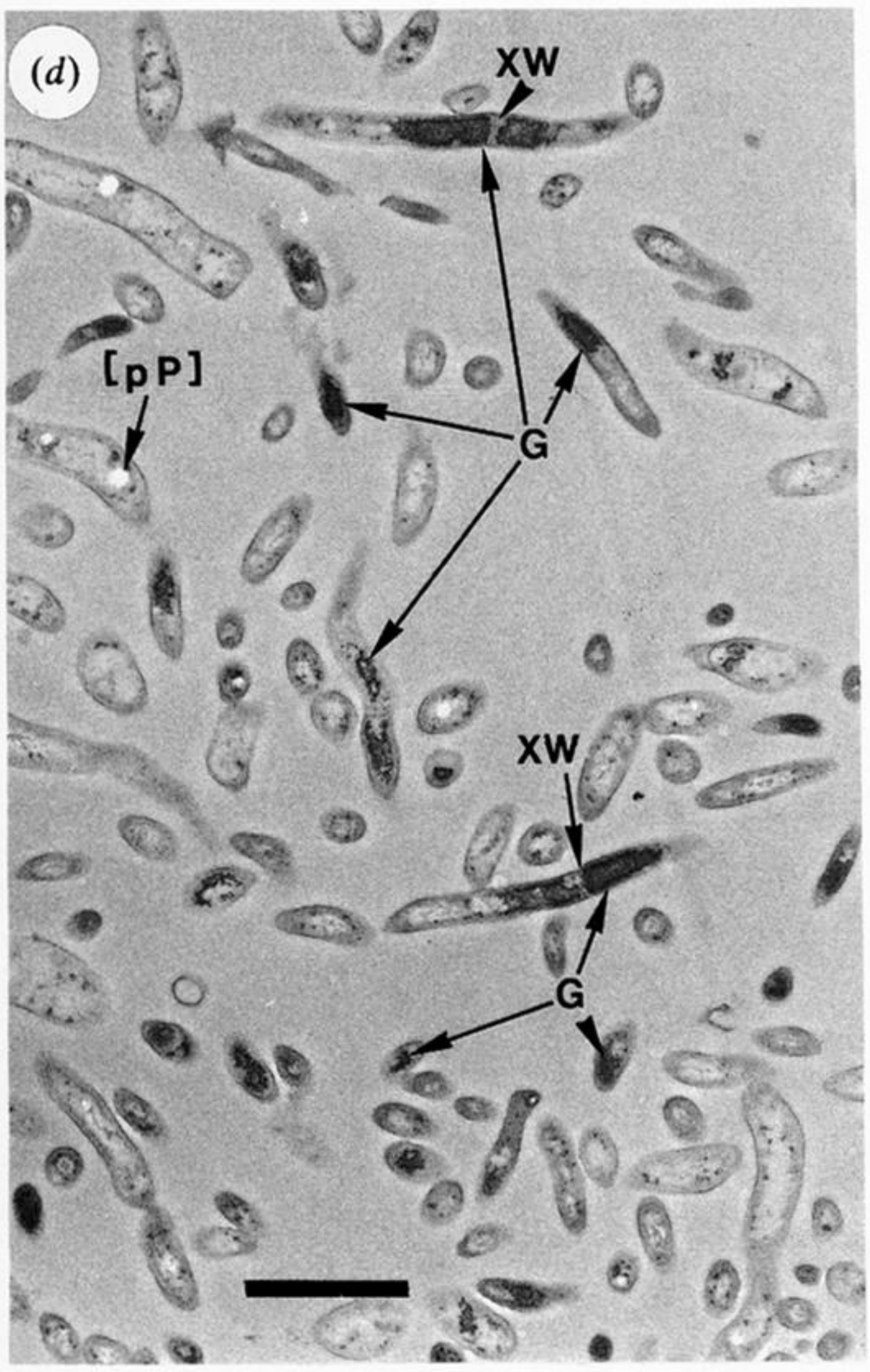


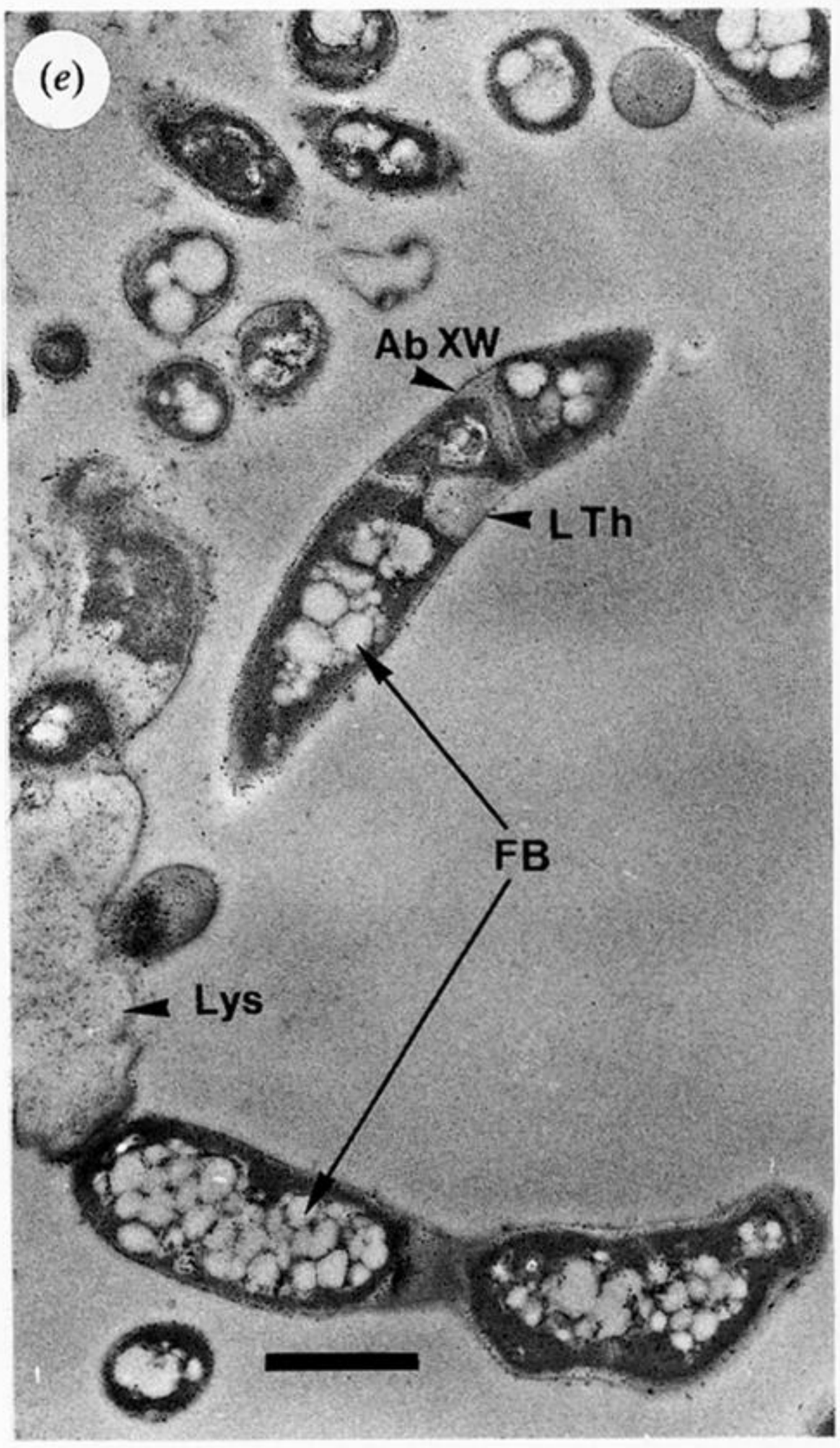
Figure 4. During growth on mannitol, a *bldA* mutant produces chains of spores showing phase II glycogen accumulation. (*a*) Upper layer of J1700, 5 days, uranyl acetate. (*b*) Upper layer of J1700, 7 days, silver proteinate. Key: Sp = spore or spore chain; [pP] = hole left by destruction of polyphosphate granule; G = glycogen; FB, fatty or oily bodies. Bar markers: 2 μm.

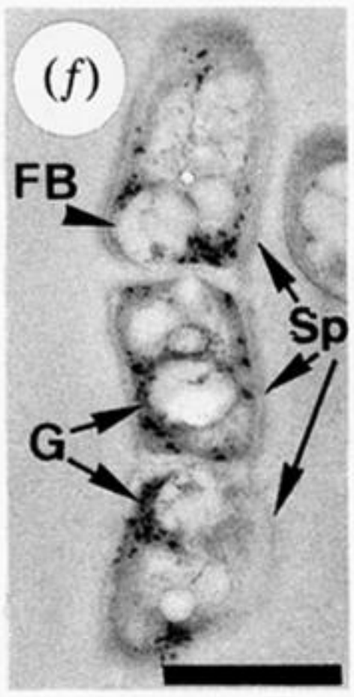












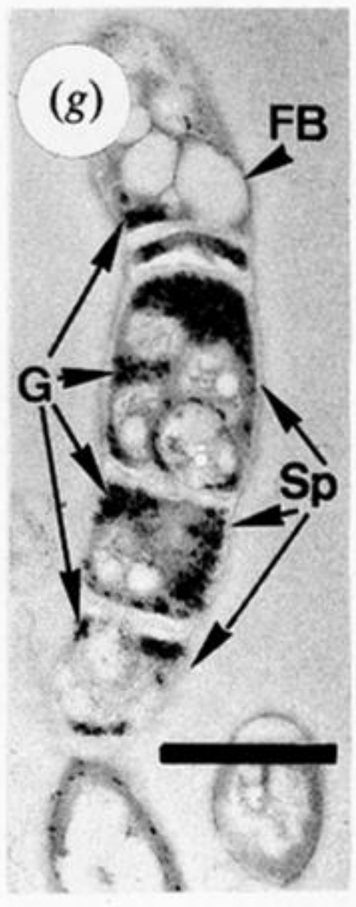
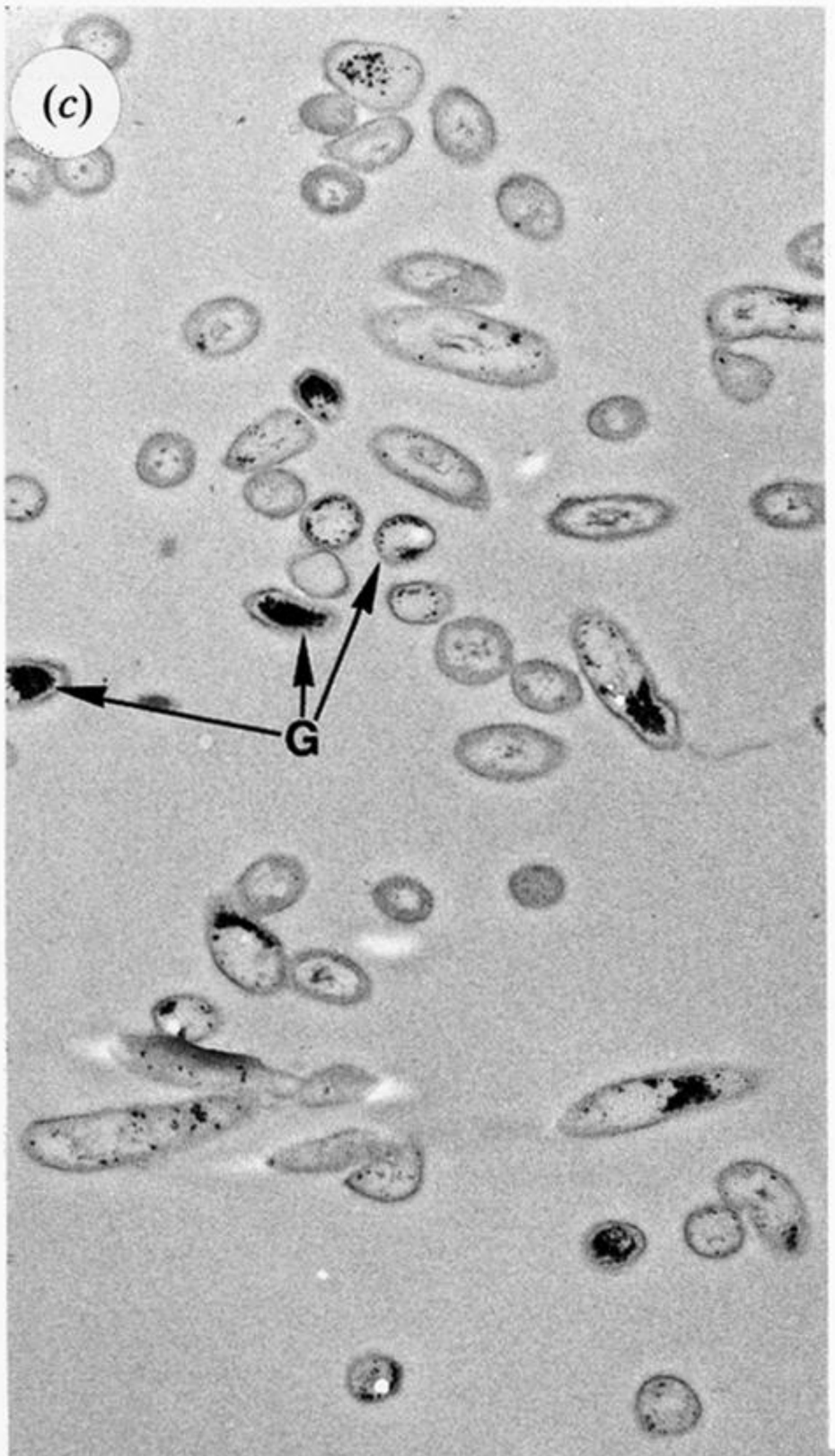
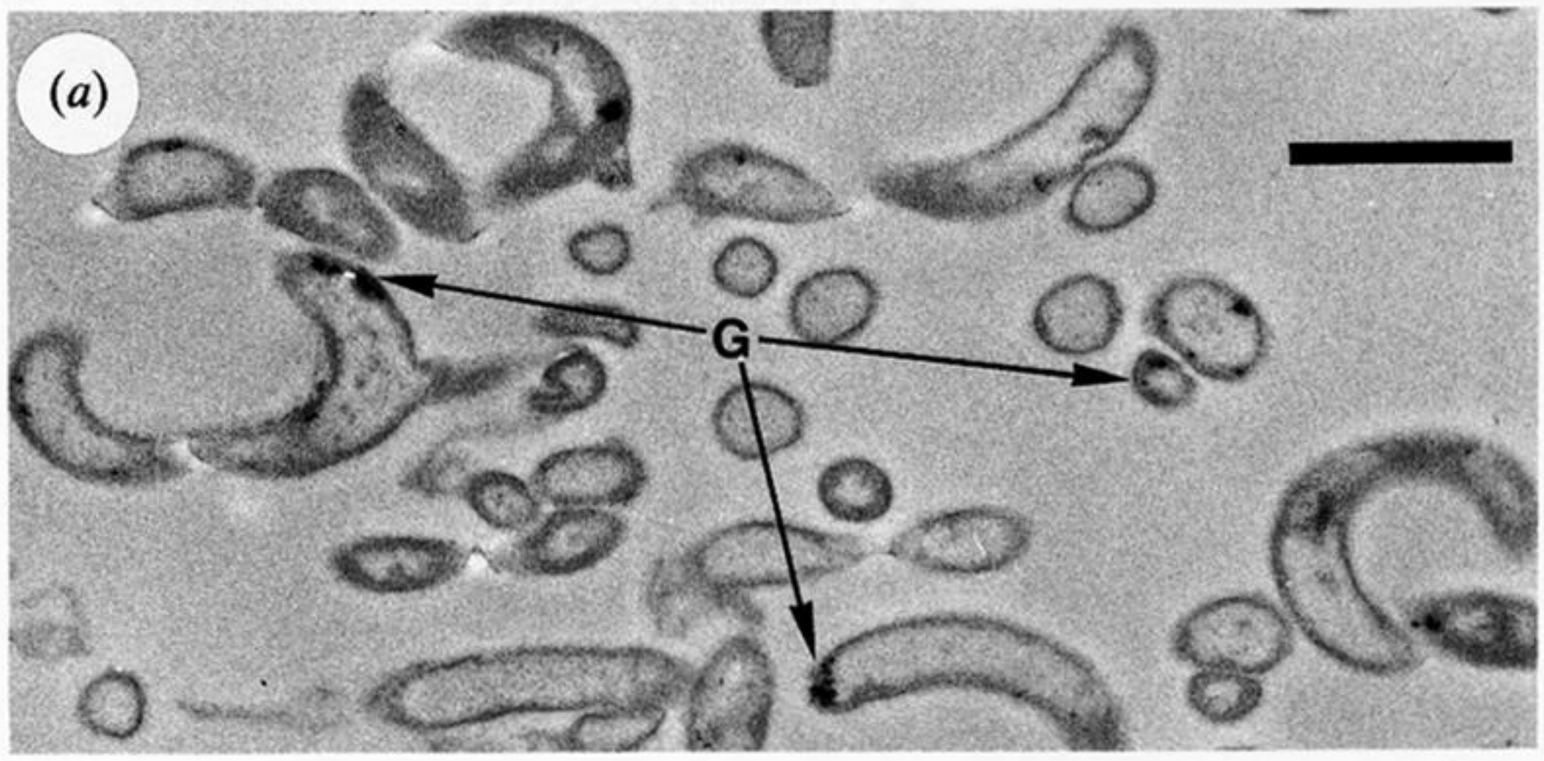
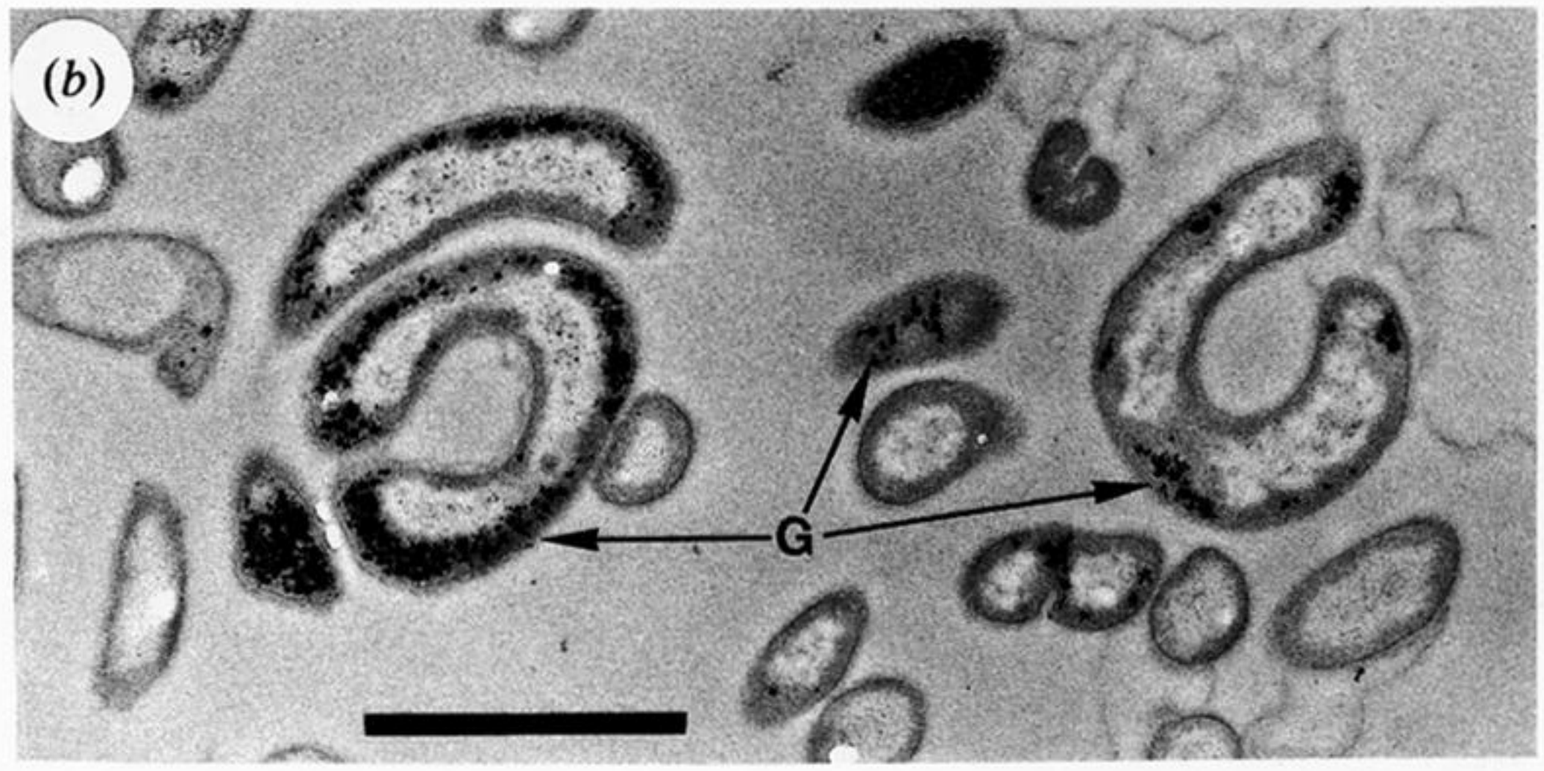
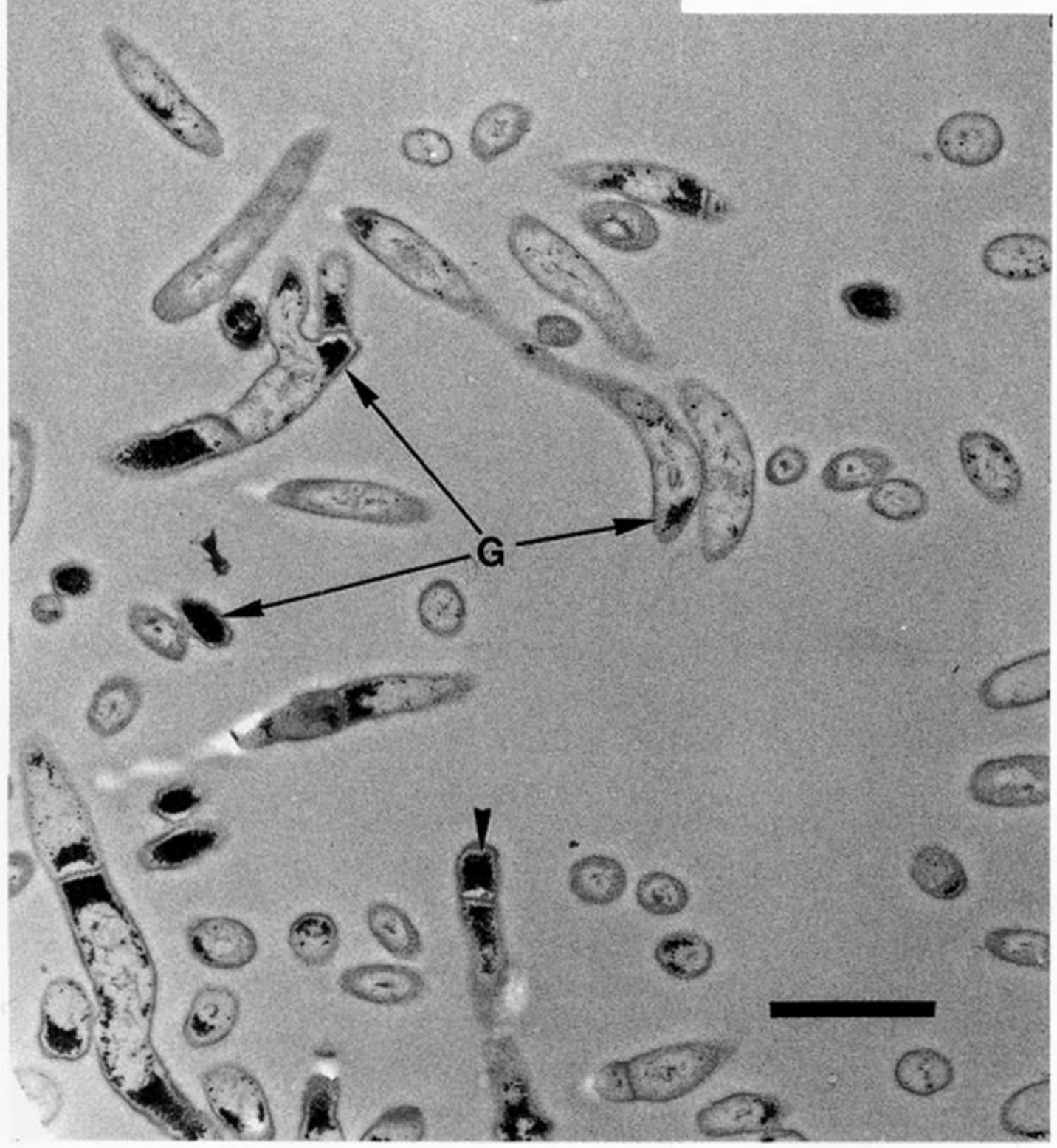


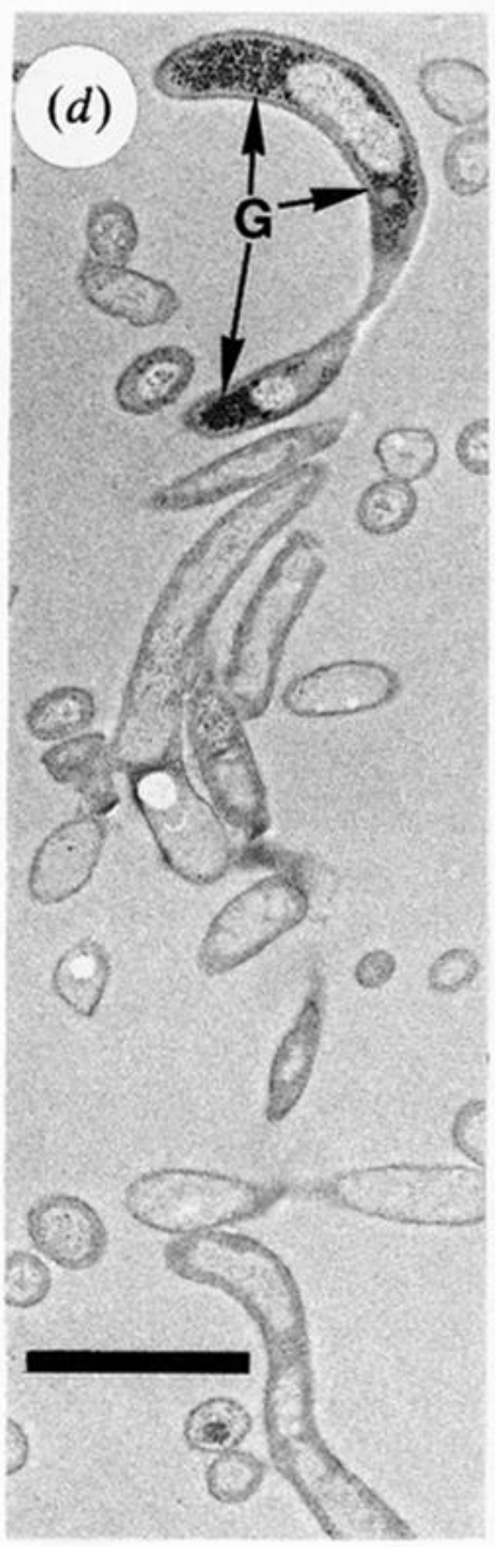
Figure 5. Glycogen accumulation in various representative *bld* mutants. All sections were stained with silver proteinate. (*a*) J669 (*bldB*), MM plus mannitol, 5 days: upper surface of culture; (*b*,*c*) J660 (*bldC*), MM plus mannitol, 5 days: (*b*), upper surface, (*c*), below the agar surface; (*d*) 166 (*bldF*), MM plus mannitol, 6 days: upper surface; (*e*) C103 (*bldG*), MM plus glucose, nitrocellulose, 45 h: upper surface; (*f*) and (*g*) C103 (*bldG*) MM plus mannitol, nitrocellulose, 45 h: aerial mycelium. Key: AbXW = aberrant crosswall; FB, fatty or oily bodies; G = glycogen; LTh = lateral thickening; Lys = lysed cell; [pP] = hole left by destruction of polyphosphate granule; Sp = spore; XW = crosswall. Bar markers: (*a*,*b*,*c*,*e*,*f*,*g*) 1 μm; (*d*) 2 μm.











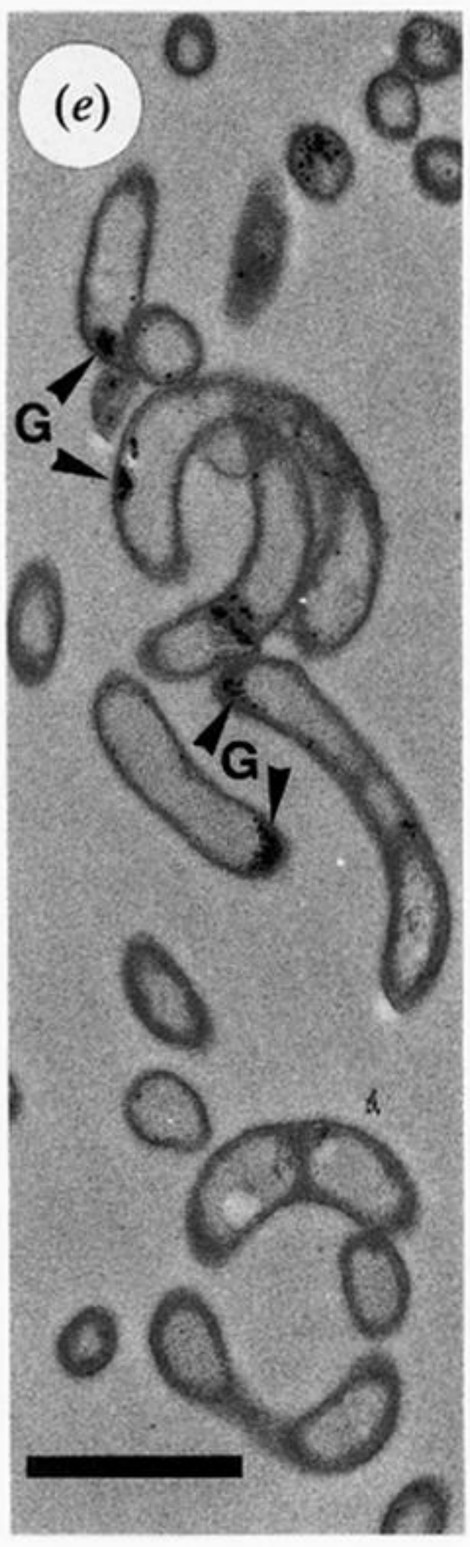


Figure 6. Glycogen deposition in the aerial hyphae of mutants (*whi*) defective in sporulation septation. All cultures were grown on mannitol and all sections were stained with silver proteinate. (*a*) C85 (*whiA85*), 4 days; (*b*) C70 (*whiB70*), 4 days; (*c*) C71 (*whiG71*), 5 days; (*d*) C119 (*whiH119*), 4 days; (*e*) C225 (*whiI225*), 4 days. Key: G = glycogen. Bar markers: 2 μm.

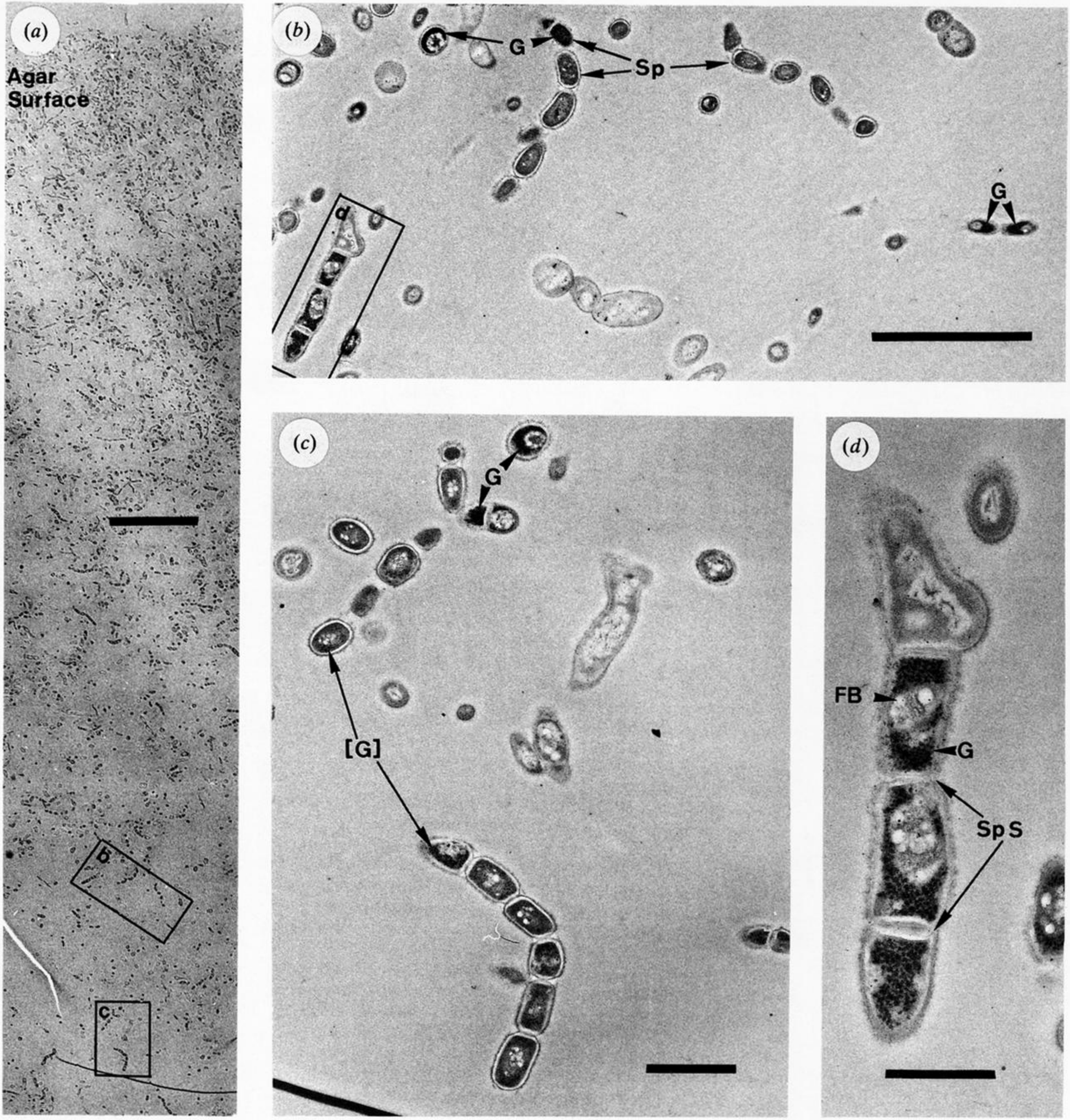
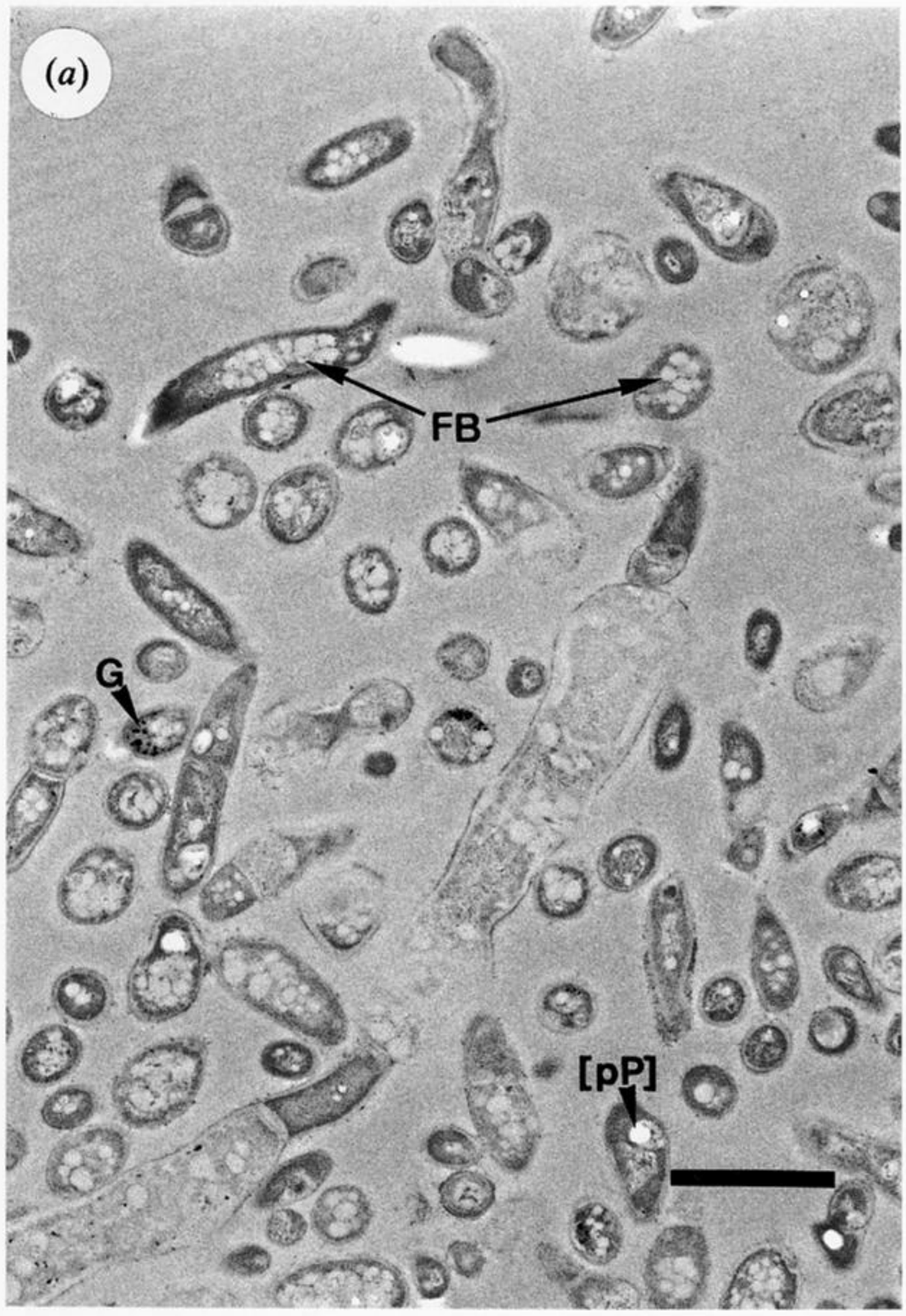
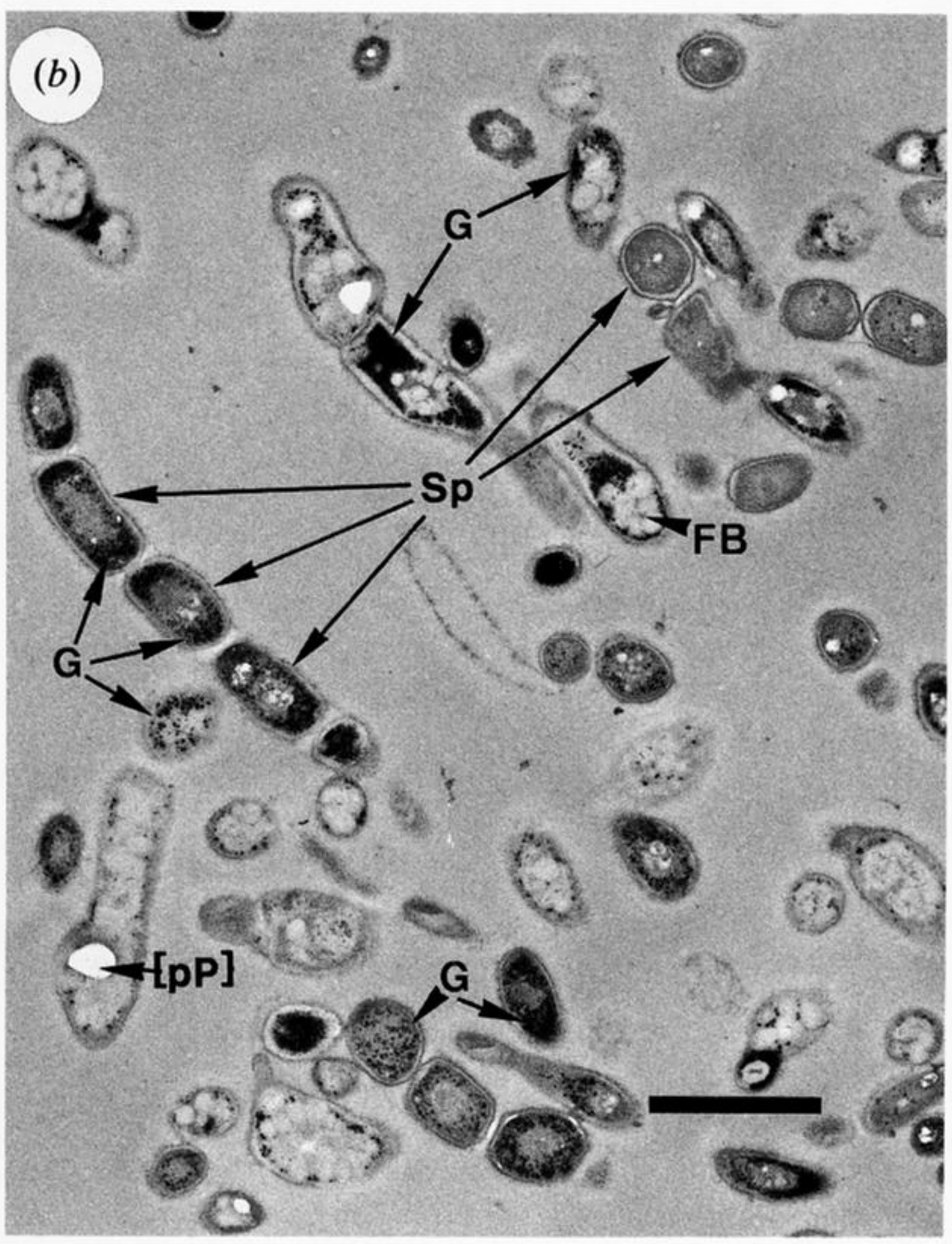


Figure 7. Glycogen synthesis in spore chains induced to develop ectopically by multiple copies of *whiG*. J1501 carrying pIJ4412 (a multicopy, *whiG*-containing plasmid) was cultured for 5 days on MM plus mannitol and stained with silver proteinate. (*a*) Vertical section through part of a colony. The position of the agar surface is indicated. Boxed areas containing spore chains are shown at higher magnification in (*b*), (*c*) and (*d*). Key: G = glycogen (brackets indicate light deposit); Sp = spore; SpS = sporulation septum; FB = fatty or oily bodies. Bar markers: (*a*) 20 μm; (*b*) 5 μm; (*c*) 2 μm; (*d*) 1 μm.





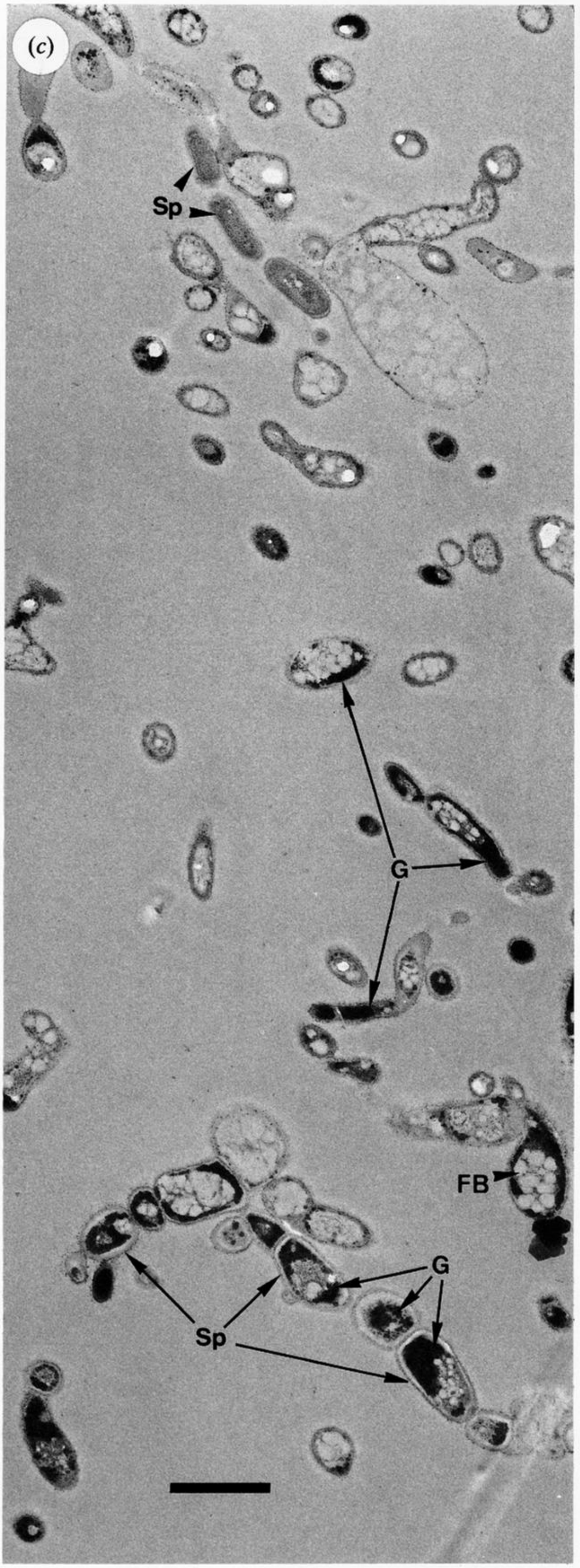
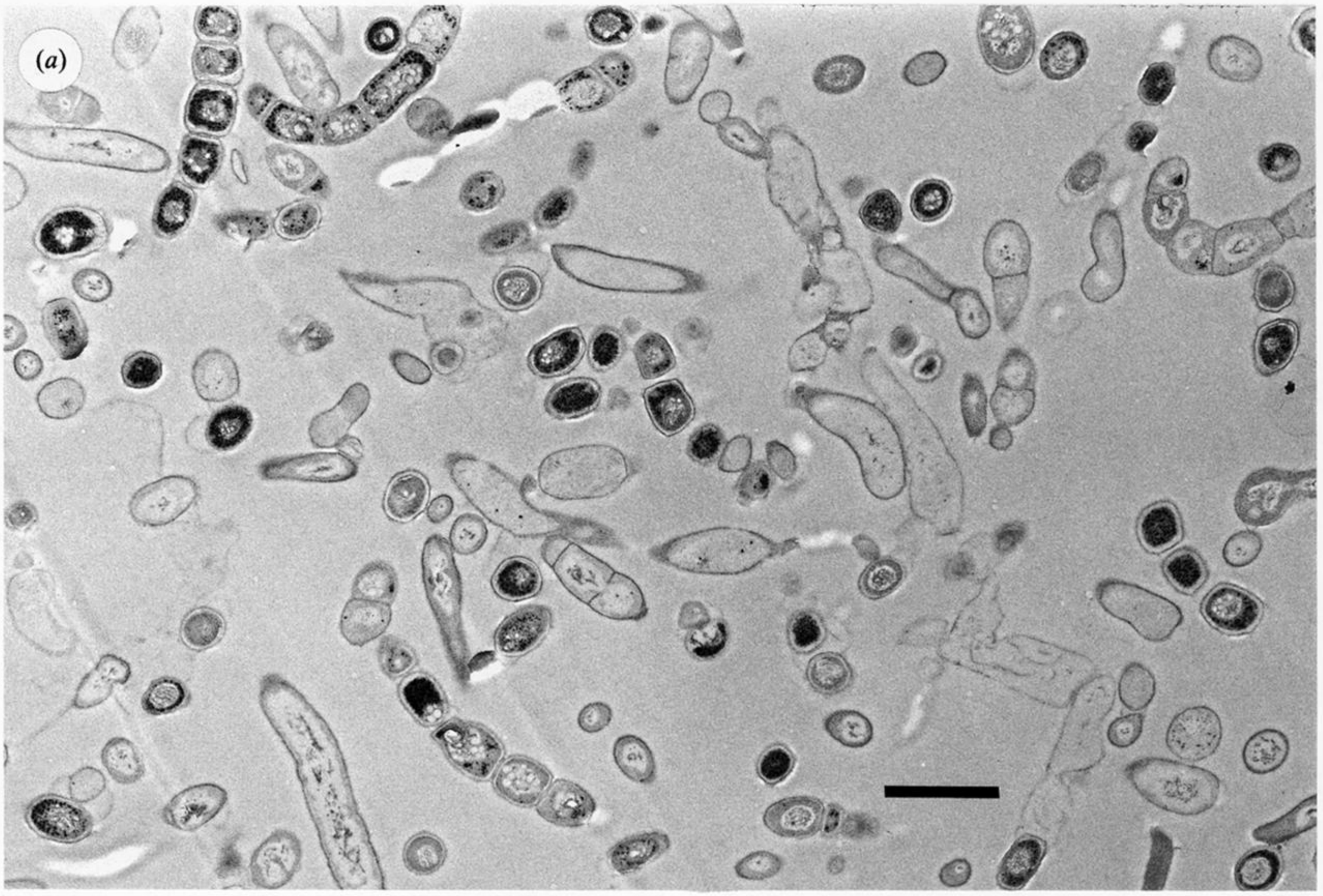
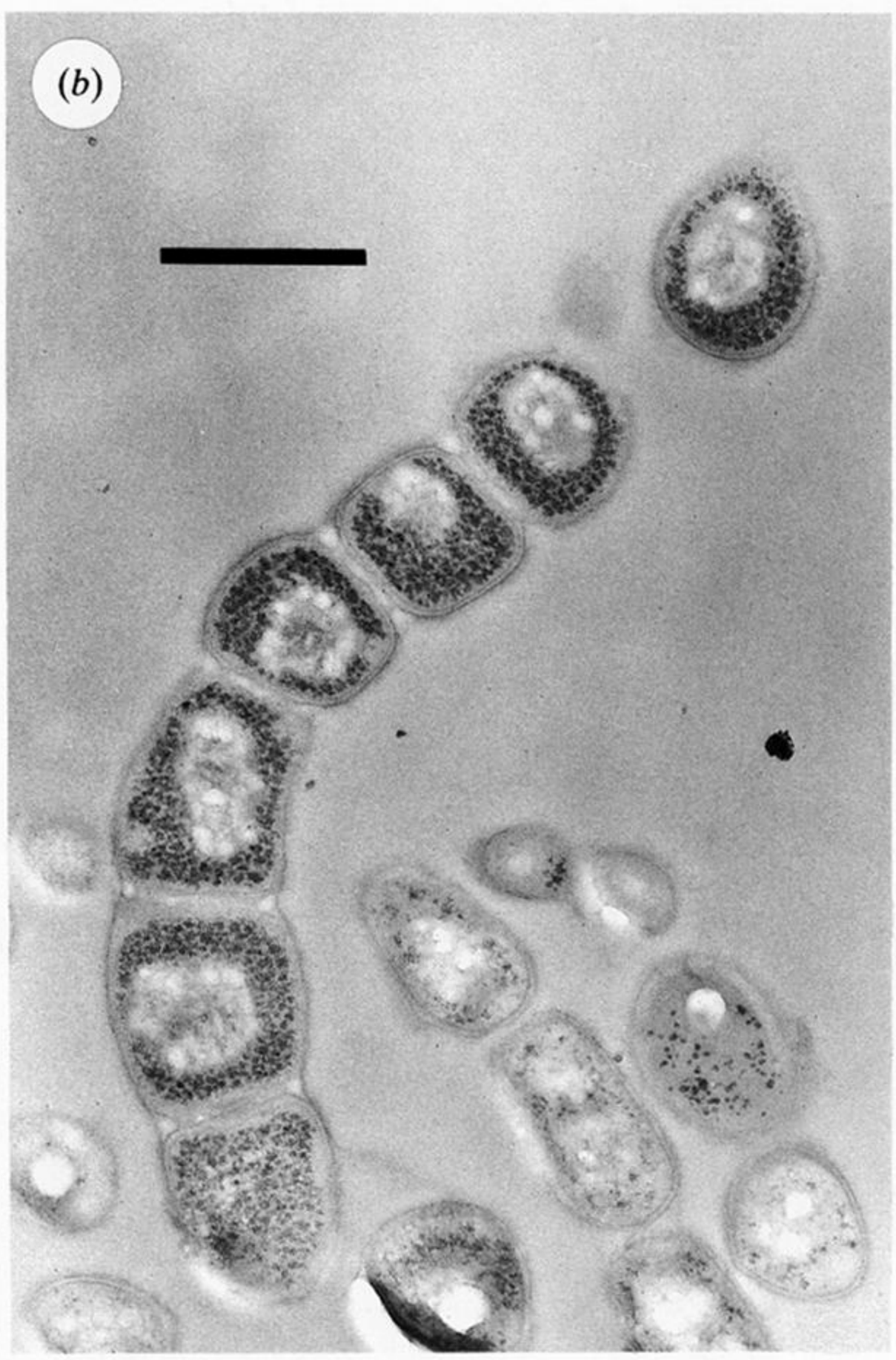


Figure 8. Medium-dependent ectopic sporulation of a *bldA* mutant induced by multiple copies of *whiG* is accompanied by glycogen deposition. The strain used was J1700 (a *bldA39* mutant) carrying pIJ4412 (a multicopy *whiG*-containing plasmid). Sections were stained with silver proteinate (*a*) 5 days, MM plus glucose (air-agar interface region); (*b*,*c*) 5 days, MM plus mannitol [(*b*): aerial mycelium; (*c*): 100 μM below the agar surface]. Key: G = glycogen granules; Sp = spore; [pP] = hole left by destruction of polyphosphate granule; FB = fatty or oily bodies. Bar markers: 2 μm.





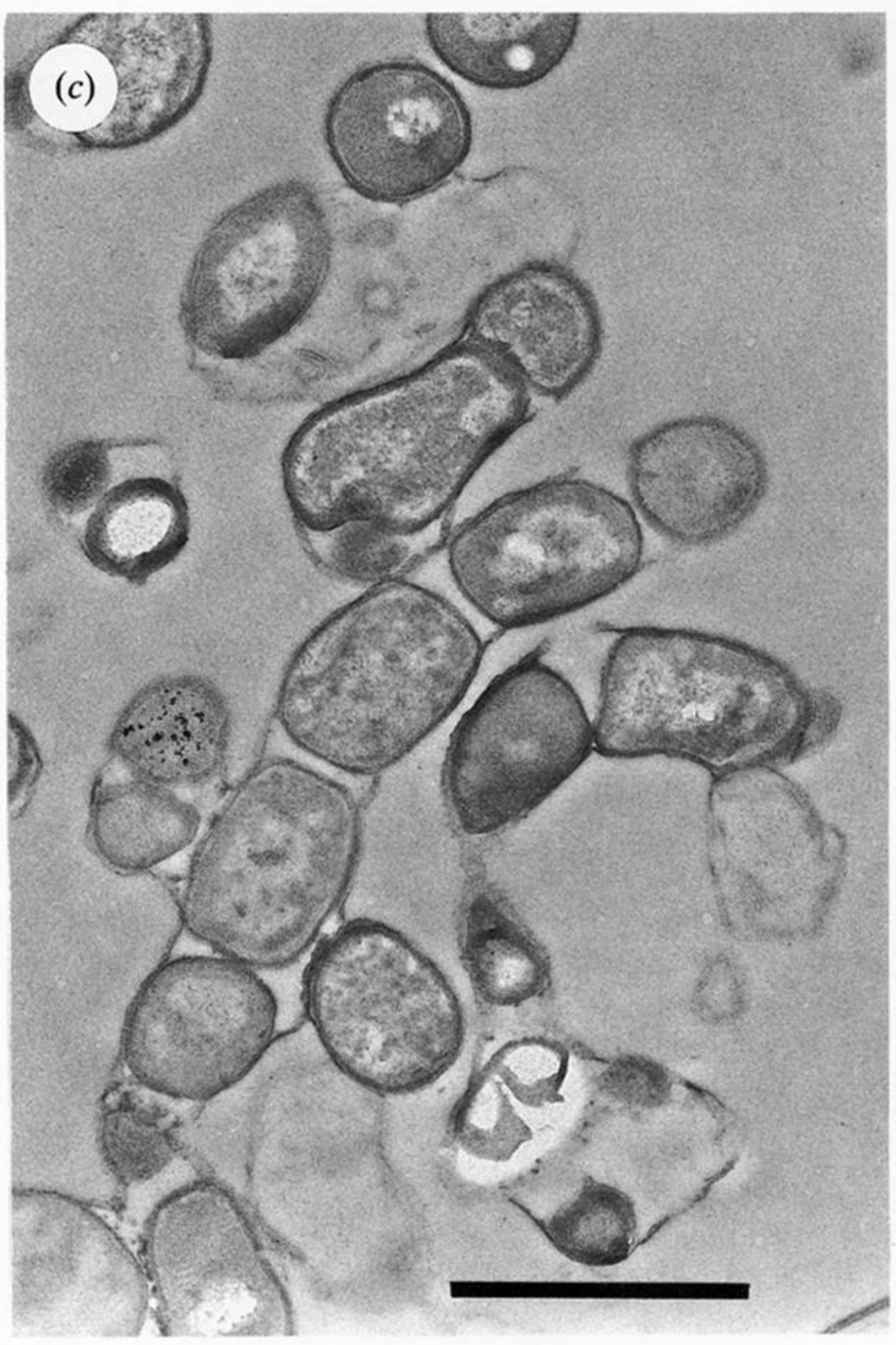


Figure 9. Effects on phase II glycogen deposition of mutations in, or extra copies of, *whiE* genes for spore pigment biosynthesis. (*a*) C107 (*whiE107*), MM plus mannitol, 4 days. Note that many well-developed spores contain very dense glycogen deposits; (*b*) C107 containing the plasmid vector pIJ922 also shows abundant glycogen deposition, MM plus mannitol, 5 days; (*c*) C107 containing extra copies of *whiE* ORFs I-VII on pIJ2156 (a pIJ922 derivative), showing almost no detectable glycogen deposits, MM plus mannitol, 4 days. Bar markers: (*a*, *c*): 2 μm; (*b*) 1 μm.