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Possible Involvement of Bacterial Autolytic Enzymes in Flagellar Morphogenesis

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Autolytic enzymes were found to be required for flagellar morphogenesis in *Bacillus subtilis* 168 and *Bacillus licheniformis* 6346. Two previously characterized, poorly lytic, chain-forming mutants of *B. subtilis* 168, strains FJ3 (temperature conditional) and FJ6, each 90 to 95% deficient in the production of *N*-acetylmuramyl-L-alanine amidase and endo-β-*N*-acetylglucosaminidase, were observed to be nonmotile at 35°C in a variety of liquid and semisolid media. In contrast, cells of the isogenic wild-type strain were motile and fully separated. Electron microscopy revealed the complete absence of flagella on the mutant cells. Similar observations were made with another poorly lytic strain of *B. subtilis* 168 (Nil5) and with two poorly lytic, phosphoglucomutase-deficient mutants of *B. licheniformis* 6346 (MH-3, MH-5). In minimal media lacking galactose (restrictive conditions), the *B. licheniformis* mutants failed to form flagella, or had serious abnormalities in flagellar morphogenesis and motility. Under permissive conditions, mutants FJ3 (grown at 17°C) and MH-5 (grown with added galactose) showed increased autolytic activities, grew in the dechained form, and regained their capacities to synthesize functional flagella. Examination of several classes of spontaneous revertants derived from the various mutant strains further demonstrated a close relationship between autolysin activity and flagellation in the two *Bacillus* spp.

Autolysins are a group of cell wall-degrading enzymes which occur widely in bacteria (22, 38). These enzymes hydrolyze specific chemical bonds in the peptidoglycan component of the procaryotic cell wall. They have a variety of specificities (38), and several have been purified to homogeneity (for examples, see ref. 21, 23). Under conditions of unbalanced growth (34), autolysins can bring about the dissolution of a bacterial cell, a phenomenon known as autolysis. The regulation of these potentially lethal bacterial enzymes has been the subject of recent investigations (10, 21, 23).

Vegetative cells of *Bacillus subtilis* 168 and *Bacillus licheniformis* 6346 synthesize two autolysins, an *N*-acetylmuramyl-L-alanine amidase, the major autolysin, and a protease-sensitive endo-β-*N*-acetylglucosaminidase (9, 14, 21, 33). From genetic and biochemical work, there is strong evidence implicating either one or both of these enzymes in the turnover of cell wall peptidoglycan during growth (16, 30, 31), in the separation of daughter cells at the end of cell division (12, 13, 15), and in mediating the killing action of cell wall antibiotics (12, 28, 32). Similar physiological roles have been reported for the autolysins of several other bacteria (for review, see 12).

Ward (41) proposed that the endo-β-*N*-acetylglucosaminidases of *B. subtilis* 168 and *B. licheniformis* 6346 may play a role in determining the average length of the glycan strands in the peptidoglycans of these bacteria. The autolysins of these bacteria, however, do not appear to be essential for growth, since mutants of each having negligible autolytic activities have been isolated and were shown to grow with normal doubling times (12, 13). Other work has suggested that the autolytic enzymes of bacilli may also participate in sporulation, spore germination, and DNA-mediated transformation (1, 12). None of these functions, however, was found to be impaired in the poorly lytic mutants of *B. subtilis* 168 characterized by Fein and Rogers (12). These mutants are 90 to 95% deficient in the production of both vegetative autolysins. Some recent work suggests that several nonvegetative, autolysin-like enzymes may be involved in the spore-related events (9, 18, 19).

The purpose of this paper is to report an additional physiological function for autolysins in *B. subtilis* 168 and *B. licheniformis* 6346.

Previously Fein and Rogers (12) presented preliminary work suggesting that their poorly lytic *B. subtilis* 168 mutants were nonmotile. In the present study, it will be shown that these mutants and other poorly lytic mutants of *B. subtilis* 168 and *B. licheniformis* 6346 are defective in flagellar morphogenesis. A possible model for the involvement of autolysins in flagellar morphogenesis will be discussed.

(This work was presented in part at the 28th Annual Meeting of the Canadian Society of Microbiologists in Montreal [J. E. Fein, Abstr. Annu. Meet. Can. Soc. Microbiol. 1978, M11p].)

MATERIALS AND METHODS

Organisms, media, and cultural conditions. The strains of *B. subtilis* 168 and *B. licheniformis* 6346 used in this investigation are listed in Table 1 along with their genotypes or phenotypes, derivations, and sources. The cultural conditions and maintenance of stock cultures were as previously described (12, 13). Growth was monitored by absorbancy measurements at 675 nm in a Bausch and Lomb Spectronic 70-4 spectrophotometer. Deviations from Beer's law were corrected as by Toennies and Gallant (39).

For the experiments, the following media were used: Penassay broth (Pen B); Spizizen minimal medium (36) supplemented by $MnSO_4 \cdot 4H_2O$ (0.01 μg/ml) and, where indicated, by sodium L-glutamate (0.3%) (SMM); and a minimal medium without added citrate (MSM [12]). This was sometimes supplemented by L-arginine (0.2%). Glucose, glycerol, or glucose-free galactose (Sigma Chemical Co.) were added as sterile solutions to the sterilized, cooled basal media at a final concentration of 0.5%. Amino acids (20 μg/ml) and thymine (50 μg/ml) were also added where necessary, to meet the auxotrophic requirements of the strains. Sandwich plates containing Procion brilliant red-stained walls from *B. subtilis* 168trp were prepared as previously described (12). These were used in the preliminary screening of the *B. subtilis* mutants and revertants for autolysin production. On this medium, poorly lytic strains are distinguished by the absence of zones of clearing around their colonies after anaerobic treatment (13).

For the motility studies and the isolation of revertants, the following semisolid media were employed: nutrient gelatin agar (NGA [27]); Pen B solidified with 0.3 to 0.5% agar (Pen A); SMM glycerol plus L-glutamate, solidified with 0.3% agar (SGGA); and MSM glycerol (MG) containing L-arginine, citrate (0.1%), and 0.4% agar. The method for obtaining spontaneous revertants of Lyt⁻ mutants on semisolid motility media has been described (12).

Detection of motility. Bacteria cultured in liquid media were examined at different stages of growth for motility, by phase-contrast or dark-field microscopy. The spreading of the strains on semisolid motility

TABLE 1. *Bacterial strains*

Strain	Genotype or phenotype	Source and/or reference
B. subtilis 168		
168trp	*trpC2*	J. Spizizen
FJ3	*metC3* Lyt1 (temperature sensitive)	(12)
FJ6, FJ7	*metC3* Lyt2	(12)
FJ8	*metC3*	(12)
FJ16, FJ17, FJ18	*metC3*	Spontaneous, from FJ3 (12)[a]
FJ23	*metC3*	Spontaneous, from FJ7 (12)[a]
FJ60	*trpC2 thyA thyB* PBSX⁻[b]	Spontaneous, from Nil5[a]
Nil5	*trpC2 thyA thyB* PBSX⁻ Lyt⁻	D. Karamata
B. licheniformis 6346		
MH-1	His⁻	(13)
MH-3	His⁻ Pgm⁻[c]	(13)
MH-5	His⁻ Spo⁻ Pgm⁻[c]	(13)
Spontaneous derivatives of MH-3		
MH-3R1 to MH-3R9, inclusive	His⁻	(13)[d]
MH-3R10	His⁻	This study[a]
Spontaneous derivatives of MH-5		
MH-5C	His⁻ Spo⁻ Pgm⁻[e]	C. W. Forsberg[f]
MH-5R1, MH-5R2	His⁻ Spo⁻	(13)
MH-5R3	His⁻ Spo⁻ Pgm⁻[e]	This study[a]
MH-5R4, MH-5R5	His⁻ Spo⁻	This study[a]
MH-5R6	His⁻ Spo⁻	This study[d]

[a] Selected for motility on a semisolid medium (Pen A, NGA, or SGGA).
[b] Noninducible for defective phage PBSX (31).
[c] These phosphoglucomutase-deficient (Pgm⁻) strains are poorly lytic.
[d] Selected for ability to grow with galactose as a sole carbon source.
[e] These Pgm⁻ strains are phenotypically Lyt⁺.
[f] Pooley et al., Abstr. First Int. Congr. Bacteriol., Jerusalem, 2:90, 1973.

media was also determined.

Electron microscopy. Samples of bacteria at different stages of growth were fixed by gently mixing them with Formalin, added to a final concentration of 5%. The fixed samples were then dried on Parlodion and carbon-coated grids and negatively stained with 1% phosphotungstic acid at pH 7.2. In some cases, 0.1% phosphotungstic acid was used. The stained bacteria were examined for the presence of flagella with a Philips EM 300 electron microscope.

Measurement of lytic enzymes in native walls and in LiCl extracts. *B. subtilis* cell extracts containing autolytic *N*-acetylmuramyl-L-alanine amidase and β-*N*-acetylglucosaminidase activities were prepared by suspending lyophilized, exponential-phase bacteria in 5 M LiCl (200 mg of dry cells per ml) at 0°C (7, 12). The assays for measuring the lytic activities contained in the cell extracts were as previously described (12). In brief, 0.13 ml of crude extract was added in a round cuvette (optically matched) to 3.87 ml of (i) suspensions (1 mg/ml) of sodium dodecyl sulfate (SDS)-treated cell walls of strain 168trp in 0.05 M Tris-hydrochloride buffer (pH 8.0) containing 0.01 M $MgCl_2$ (amidase assay), or (ii) suspensions (0.5 mg/ml) of *Micrococcus luteus* SDS-treated cell walls in 0.05 M sodium acetate buffer (pH 5.6), also containing 0.01 M $MgCl_2$ (β-*N*-acetylglucosaminidase assay [11]). With the exception of the experiment presented in Fig. 6, a Bausch and Lomb Spectronic 70-4 spectrophotometer was used to measure the lytic activities of the extracts. With this instrument, one unit of lytic activity was taken as that which lowered the absorbancy of the suspensions at 450 nm, at 37°C, by 0.001 unit per min during the period of approximately linear decrease.

For strains of *B. licheniformis* 6346, similar methods were used to prepare and assay the LiCl-solubilized autolysins (100 mg of dry cells extracted per ml of LiCl). Autolytic amidase in the extracts was determined by measuring their capacity to lyse SDS-treated cell walls from strain MH-1 suspended (1 mg/ml) in 0.025 M borate buffer (pH 9.5) containing 0.01 M $MgCl_2$ (37°C). At this pH, the β-*N*-acetylglucosaminidase of this bacterium has negligible activity (13). The glycosidase is active at pH 5.6 (13), and was measured using the *M. luteus* SDS-treated cell wall assay described above. The latter assay appears to be specific for the glycosidase, since lysis of the *M. luteus* walls by the *B. licheniformis* extracts is not accompanied by the liberation of L-alanyl-NH_2 groups, as measured by the procedure of Forsberg and Ward (14) (J. E. Fein, unpublished data).

Isolated cell walls containing endogenous autolysins (native walls) were prepared from exponential-phase cells of *B. subtilis* 168 as previously described (12), except that TL buffer (pH 7.1; 0.05 M Tris buffer containing 0.024 M LiCl) was used in the washing steps, rather than TL buffer (pH 8.0; 0.05 M Tris buffer containing 0.041 M LiCl). This was to further reduce the possibility of autolysis during the preparation of the walls, and had no effect on the subsequent lytic activity of the walls.

Native wall lysis was measured at 37°C in TL buffer (pH 8) containing 0.01 M $MgCl_2$ and in 0.1 M acetate buffer (pH 5.6) containing 0.01 M $MgCl_2$, by monitoring the diminution of extinction values of the suspensions as described above. Native walls of *B. licheniformis* 6346 were prepared in a similar manner and were assayed for autolytic activity by the method of Forsberg and Rogers (13) in 0.05 M sodium carbonate buffer (pH 9.5) and in 0.05 M potassium phthalate buffer (pH 5.6).

Miscellaneous. Phosphoglucomutase activity in extracts obtained from disrupted bacteria (grown in Pen B) was measured by the method of Forsberg et al. (15). Extracellular protease production was assayed on a skim-milk agar (12). The induction by mitomycin C of defective phage PBSX in strains of *B. subtilis* 168 and the determination of the average number of bacterial cells per chain in growing cultures by the Victoria blue method were as previously described (12). The latter values are reported in the text ± their standard deviation. All chemicals were of reagent grade and were purchased from commercial sources.

RESULTS

Motility behavior of strains FJ3, FJ6, and FJ8. Fein and Rogers (12) reported the isolation of two poorly lytic mutants of *B. subtilis* 168, each of which is grossly deficient in both the autolytic amidase and β-*N*-acetyl-glucosaminidase (strains FJ1 and FJ2). The loss of both autolysin activities is probably the consequence of a single point mutation in each mutant (12). From FJ1 and FJ2 an isogenic set of strains carrying the original *lyt-1* (strain FJ3) and *lyt-2* (strains FJ6 and FJ7) mutations was constructed by transformation (by congression [12]). An isogenic strain wild type with respect to autolytic activity was also constructed (strain FJ8). The mutants were shown to grow in a variety of minimal and rich liquid media at 35°C with normal doubling times, and to occur as long chains of unseparated but septated cells (12).

Further investigation by phase-contrast and dark-field microscopy has revealed that, regardless of their phase of growth or the medium employed (Pen B, SMM glucose ± L-glutamate, or MSM glucose ± L-arginine), the mutant cells are nonmotile (35°C). In contrast, strain 168trp and the isogenic wild-type strain FJ8 exhibited rapid motility in all media tested, especially in the latter half of exponential growth. The nonmotile behavior of the mutants could also be demonstrated on a rich semisolid medium. Typical growth patterns for the mutant and wild-type strains on NGA are shown in Fig. 1.

The wild-type and mutant strains were grown in Pen B at 35°C. Samples of the bacteria at different stages of growth were prepared for electron microscopy, and the cells were examined for the presence of flagella. Typical electron micrographs are shown in Fig. 2 A to C. Unlike the wild-type strain, which was peritrichously flagellated, no detectable flagellar structures

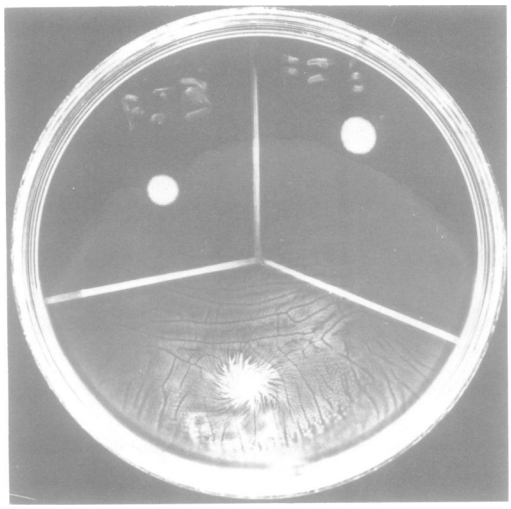

FIG. 1. *Spreading behavior of B. subtilis 168 Lyt⁺ (FJ8) and Lyt⁻ (FJ3 and FJ6) strains on NGA. The plates were photographed after 14 h of incubation at 35°C. During the short incubation period, strain FJ8 (lower center) spread rapidly over the surface of the agar. In contrast, the two poorly lytic mutants, FJ3 (upper left) and FJ6 (upper right), were nonmotile, and each grew in situ at its site of innoculation.*

were ever observed on any of the poorly lytic mutants.

Strong supporting evidence for the involvement of autolytic enzymes in flagellar morphogenesis came from studying the effects of temperature upon strain FJ3 and from reversion studies. It was previously reported that mutant strains carrying the *lyt-1* mutation (FJ1 and FJ3) are temperature conditional for the production of both the autolytic amidase and glycosidase. When cultured in Pen B at 17°C, these strains partially regain both autolytic activities and grow as singlets, doublets, and very short chains (12). At the low permissive temperature, many of the cells also synthesized peritrichous flagella (Fig. 2D) and exhibited normal motility. A similar temperature effect was not observed for strains carrying the *lyt-2* mutation.

Spontaneous revertants were derived from the nonmotile Lyt⁻ mutants FJ3, FJ6, and FJ7 by plating the mutants onto semisolid Pen A and selecting for cells capable of spreading growth at 30°C. By this technique, a collection of over 30 spontaneous revertants was obtained.

When cultured in Pen B at 30°C, each of the revertants grew as motile, peritrichously flagellated, single and paired cells. A typical revertant is shown in Fig. 2E. None of the revertants showed any tendency to form chains. All were Met⁻, and each appeared phenotypically Lyt⁺ when tested at 30°C on Procion-stained cell wall agar plates. Preliminary work suggests that a sizable proportion (approximately 25%) of the revertants derived from strain FJ3 are temperature conditional, being phenotypically Lyt⁺ at 42°C. Four nonconditional revertants (strains FJ16, FJ17, FJ18, and FJ23) have been shown to produce high levels of both autolytic amidase and β-*N*-acetylglucosaminidase (12).

Motility behavior of strain Nil5 of B. subtilis 168. Pooley and co-workers (31, 33) recently studied a poorly lytic mutant of *B. subtilis* 168 (strain Nil5) that is defective in phage PBSX induction and deficient in peptidoglycan turnover during growth. The strain was noted to grow with normal doubling times in liquid culture, occurring as nonmotile cells in short chains.

The motility and autolytic characteristics of strain Nil5 have been investigated further. In confirmation of Pooley's observations, strain Nil5 was observed to grow as nonmotile chains (11.1 ± 4.6 cells per chain) in Pen B supplemented with thymine. The lack of motility was also demonstrated by the inability of the mutant to spread on NGA plus thymine. When examined under the electron microscope, the cells of the mutant appeared to be completely devoid of flagella, regardless of cell chain length (Fig. 2F and G). Lithium chloride extracts of Nil5 contained reduced levels of both *B. subtilis* autolysins (Fig. 3). The levels of these enzymes were higher than those previously reported for mutants FJ3 and FJ6 (12). Native walls of Nil5 lysed at pH 8.0 and pH 5.6 at approximately 22 and 32% of the rates for native walls prepared from the wild-type strain, respectively (Fig. 4).

A spontaneous revertant of strain Nil5 (strain FJ60) was isolated on NGA plus thymine. When cultured in Pen B containing thymine, this revertant grew as motile singlets and doublets, having numerous peritrichous flagella (1.8 ± 0.6 cells per chain). The morphology of the strain is shown in Fig. 2H. Like Nil5, the revertant was found to be phenotypically Spo⁺, Trp⁻, and Thy⁻ and deficient in the production of phage PBSX. Lithium chloride extracts prepared from strain FJ60 were highly active in both the autolytic amidase and β-*N*-acetylglucosaminidase assays (Fig. 3). In addition, native walls prepared from the revertant lysed faster than those of the wild-type strain, at pH 8.0 and pH 5.6 (Fig. 4). These quantitative differences have not been investigated further. These findings for strains Nil5 and FJ60 are in agreement with those obtained for the other Lyt⁻ and revertant strains of *B. subtilis* 168 reported above, and serve to further demonstrate the existence of a close relationship between autolytic activity and flagellar morphogenesis in this species.

Motility behavior of poorly lytic strains

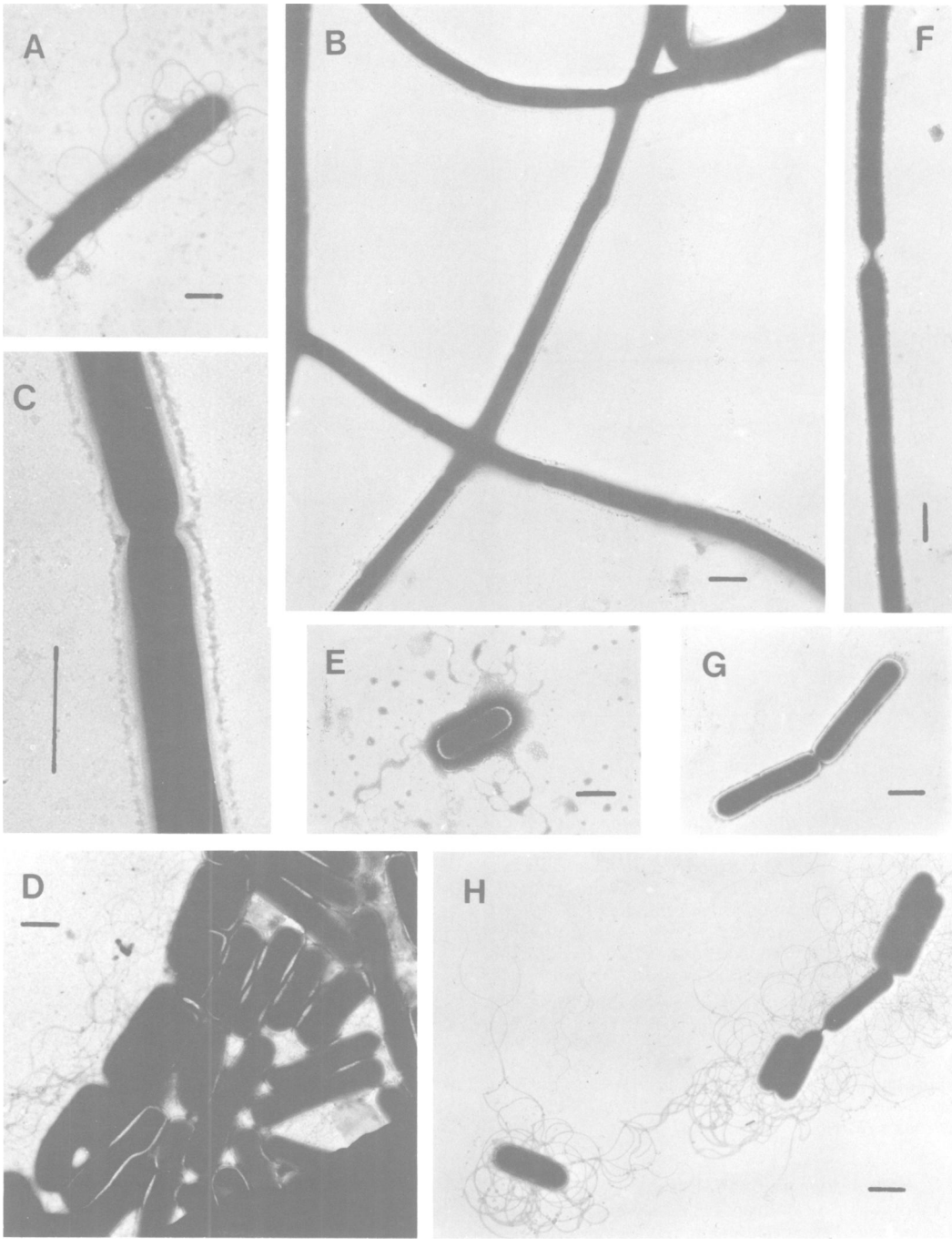

FIG. 2. *Morphology of wild-type, Lyt⁻, and Lyt⁺ revertant strains of B. subtilis 168 grown past midexponential phase in Pen B. The cells were negatively stained with phosphotungstic acid and examined under an electron microscope for flagella. The bar indicates 1 μm. Electron micrographs of the wild-type strain FJ8 (A), Lyt⁻ chain-forming mutants FJ3 (B) and FJ6 (C), and revertant FJ16 (E), grown at 35°C; mutant FJ3 (D) grown at the permissive temperature for autolysin synthesis (17°C); mutant Ni15 (F, G) and its revertant FJ60 (H) grown at 35°C. Only those cells having high autolytic activities were observed to form flagella (A, D, E, and H).*

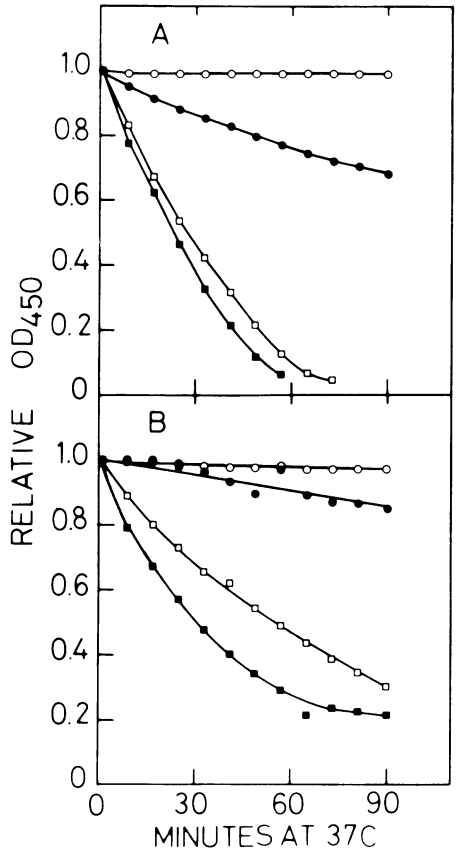

FIG. 3. *Lysis of B. subtillis 168 and M. luteus SDS-treated cell walls with crude LiCl extracts made from strains FJ8, Ni15, and FJ60. The extracts were obtained from lyophilized, exponential-phase cells as described in the text. (A) Lysis of B. subtilis 168 cell walls at pH 8 in TL buffer containing 0.01 M $MgCl_2$ (amidase assay). Symbols: (○) control (no added autolysins); (□) FJ8; (●) Ni15; (■) FJ60. Rough approximations of the activities for wall lysis were 18.8, 4.4, and 21.7 units for the extracts derived from strains FJ8, Ni15, and FJ60, respectively. (B) Lysis of M. luteus cell walls at pH 5.6 in 0.05 M acetate buffer containing 0.01 M $MgCl_2$ (β-N-acetylglucosaminidase assay). Symbols are as in (A). Approximate activities for wall lysis were 12.5 (FJ8), 1.8 (Nil5), and 22.6 units (FJ60). OD_{450}, Optical density at 450 nm.*

of *B. licheniformis* 6346. Forsberg and Rogers (13) isolated and characterized several mutants of *B. licheniformis* 6346 that have altered autolytic phenotypes. Among these were two poorly lytic, chain-forming mutants, strains MH-3 and MH-5. In each of these, the primary lesion was shown to be a defect in phosphoglucomutase (Pgm^-). As a result the cell walls of the mutants have an altered chemical composition (13, 15). The Lyt phenotype of the Pgm mutants appears to be a consequence of both the resistance of the altered mutant walls to the action of the *B. licheniformis* autolytic amidase, and the reduced production of the amidase itself (13, 15). Although the exact interrelationship between the phosphoglucomutase defect and the reduced synthesis of amidase is not understood, evidence has been presented that suggests that only a single mutation is responsible for the observed phenotype in each strain (13, 15).

The motility characteristics of the two Pgm^- mutants and the parental strain (MH-1) were examined and compared. In MG medium at 35°C, mutants MH-3 and MH-5 grew as nonmotile chains (37.1 ± 21.9 and 66.6 ± 48.1 cells per chain, respectively). The parent strain in the same medium grew as motile, peritrichously flagellated single and paired cells (Fig. 5A). The observed motility was most pronounced during the latter half of exponential growth. For strain MH-3, no flagella or motile cells were ever ob-

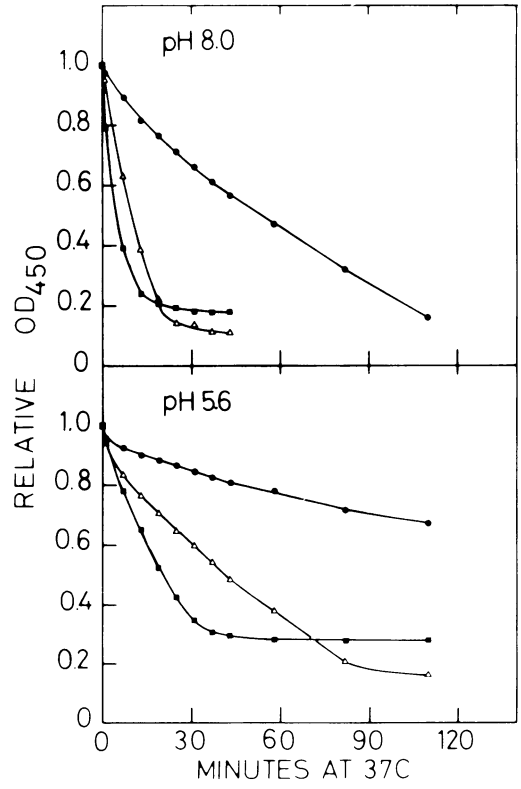

FIG. 4. *Comparative autolysis at pH 8 (top) and pH 5.6 (bottom) of native walls isolated from wild-type strain B. subtilis 168trp (△), mutant Ni15 (●), and revertant FJ60 (■). Rough approximations of the wall lytic activities for strains 168trp, Ni15, and FJ60 were 37.8, 8.4, and 70.0 units at pH 8 and 7.5, 2.4, and 15.8 units at pH 5.6, respectively.*

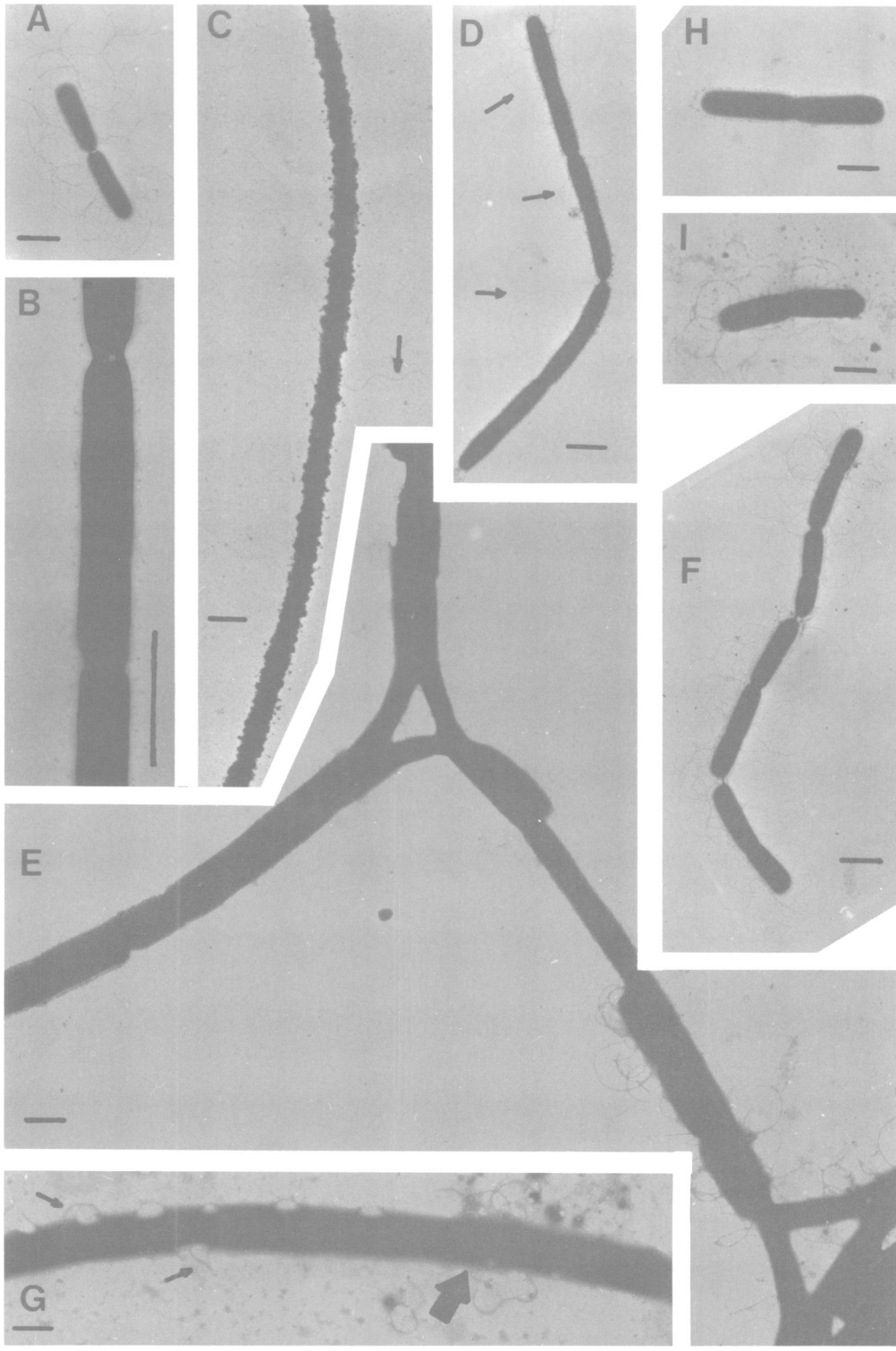

FIG. 5. *Morphology of several B. licheniformis 6346 strains grown in glycerol minimal media at 35°C. The cells were cultured past midexponential phase, then negatively stained with phosphotungstic acid and examined under an electron microscope for flagella. The bar indicates 1 μm. (A–D) Electron micrographs of cells grown in MG: (A) appearance of the parental strain MH-1, showing its peritrichous flagella; (B) part of a long, nonflagellated chain of MH-5, taken at higher magnification; (C) a long chain segment of MH-5 showing a cell with a single flagellum (arrow); (D) a short chain of MH-5, consisting of nonflagellated and sparsely flagellated cells. (E–I) Electron micrographs of cells grown in SGG: (E) sample of MH-5 culture containing chains of various sizes and degrees of flagellation; (F) a short chain of MH-5 composed of peritrichously flagellated cells; (G) part of a long, primarily nonflagellated chain of MH-5 (a few of the cells in the chain were sparsely flagellated [arrows] or had peritrichous flagella [wide arrow]; (H) nonflagellated partial revertant MH-3R2; (I) flagellated revertant MH-3R10.*

served, but for strain MH-5, an occasional sparsely flagellated cell in a chain or a motile, peritrichously flagellated single or paired cell was seen (Fig. 5B to D). The latter did not appear to be spontaneous Pgm$^-$ revertants, as tested on a galactose minimal medium (13, 15, 43).

The phosphoglucomutase deficiency in mutant MH-5 can be circumvented by growing the bacteria in media supplemented by galactose, resulting in the partial restoration of autolytic amidase activity and in the dechaining of the mutant (15). Growth of strain MH-5 in MG supplemented by galactose (0.1%) resulted in the restoration of flagellation and motility, though not to the extent characteristic of the wild-type strain. The mutant showed little tendency to chain under these growth conditions. In contrast, mutant MH-3 remained nonmotile and nonflagellated in MG containing galactose, and only partially dechained (17.1 ± 10.1 cells per chain).

Results similar to those reported above for MG with and without galactose were obtained for strains MH-1 and MH-3 grown in SMM glycerol plus L-glutamate (SGG), with and without added galactose. Flagella were never detected on the cells of mutant MH-3. The situation with MH-5 was somewhat different. In SGG lacking added galactose, a high proportion of the MH-5 cells were flagellated. Only a very low proportion of the mutant bacteria, however, exhibited motility. As with the MG-grown bacteria, the cells in the shorter chains were more likely to be flagellated than those in the longer chains (9.7 ± 9.4 cells per chain), although it was not uncommon to find some flagellated cells in the larger chains (Fig. 5E to G). A small number of motile peritrichously flagellated separated bacteria were always observed in the MH-5 cultures. Unlike the parental strain MH-1, whose swimming pattern resembled the random walk pattern described for *Escherichia coli* (4), most of the motile mutant cells appeared to swim about slowly and aimlessly. Their pattern appeared uncoordinated, with the cells often continuously tumbling, spinning, wobbling, or swimming in circles. The abnormal motility was confirmed using a semisolid medium of a composition similar to SGG (SGGA). Unlike the parental strain, the mutant failed to spread on this medium. There was no evidence for spontaneous Pgm$^-$ revertants in any of these experiments.

The addition of galactose (0.1%) to SGG resulted in the dechaining of strain MH-5 (1.9 ± 1.0 cells per chain) and in the restoration of normal motility in a large proportion of the mutant cells. The latter was confirmed by the observed spreading behavior of the mutant grown in semisolid SGGA containing galactose.

It is not obvious why the extent of flagellation in MH-5 was that much more extensive for SGG versus MG-grown bacteria. The two minimal media have basically similar compositions, differing primarily by the presence of citrate and L-glutamate and the absence of added iron in SGG. The *B. licheniformis* strains used here were observed to grow more readily (with shorter lag periods) in SGG than in MG, although the differences in the measured doubling times for exponential-phase cells were not great (Table 2).

Lithium chloride extracts prepared from exponential-phase cells of MH-5 grown in each of the minimal media were found to contain significantly reduced levels of both autolytic amidase and β-*N*-acetylglucosaminidase (Fig. 6 to 8). Similar results were obtained for MH-3 grown in SGG (Fig. 7 and 8). From the lysis data for MH-5, it would appear that the autolysin levels may be lower in the MG than in the SGG-grown cells. This, however, requires further investigation. If correct, the differences in lytic activities might explain the observed differences in the degrees of flagellation for the mutant bacteria grown in the two media. Native walls prepared from MH-5 grown in MG were also observed to lyse poorly at pH 5.6 and pH 9.5.

Forsberg and Rogers (13) previously reported that the levels of β-*N*-acetylglucosaminidase were normal in native walls prepared from strains MH-3 and MH-5, based on reducing power measurements of autolyzing walls. The differences between the results presented here for the level of the glycosidase in MH-5 and those by Forsberg and Rogers may be a consequence of the different assay procedures used.

As shown in Fig. 7 and 8, both autolysin activities were significantly increased in cells of MH-5 grown in SGG containing added galactose (0.1%). The results for the amidase are in close agreement with the previously reported findings of Forsberg et al. (15) for native wall lysis of MH-5 grown with galactose. Surprisingly, the LiCl extracts of the mutant contained approximately twice as much β-*N*-acetylglucosaminidase activity as that found in extracts of the

TABLE 2. *Growth rates of MH-1 and MH-5 in SGG and MG at 35°C*

Strain	Average doubling time (min)	
	SGG	MG
MH-1	83	70
MH-5	90	95

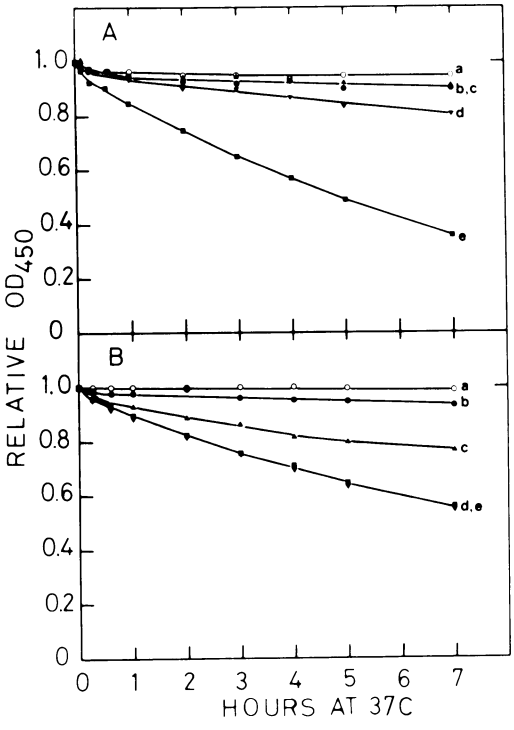

FIG. 6. *Lysis of B. licheniformis 6346 and M. luteus SDS-treated cell walls with crude LiCl extracts made from strains MH-1, MH-5, MH-5C, and MH-5R3. The LiCl-solubilized autolysins were prepared from cells grown in MG medium and were assayed as described in the text. Twice the usual amount of extract (0.27 ml) was added to the lysis tubes. A Unicam SP-600 was used to measure lysis. (A) Lysis of B. licheniformis 6346 (strain MH-1) walls at pH 9.5 in 0.025 M borate buffer containing 0.01 M $MgCl_2$ (amidase assay). Symbols: (a, ○) control (no extract added); (b, ●) MH-5; (c, ▲) MH-5R3; (d, ▼) MH-5C; (e, ■) MH-1. (B) Lysis of M. luteus walls at pH 5.6 in 0.05 M acetate buffer containing 0.01 M $MgCl_2$ (β-N-acetylglucosaminidase assay). Symbols are as in (A). Overall, the relative extent of lysis by the MH-5, MH-5R3, and MH-5C extracts was 8.4, 6.6, and 24.5% of that of the MH-1 extract in the amidase assay, and 12.6, 49.7, and 105.1% in the β-N-acetylglucosaminidase assay (corrected for controls).*

wild-type strain. The high lytic activity of MH-5 grown with added galactose correlates well with the observed motility of the strain grown under these conditions. With strains MH-1 and MH-3 grown with added galactose, only slight increases were detected in the activities of the two autolytic enzymes. The failure to stimulate the autolysins in MH-3 would probably explain the inability of the mutant cells to form flagella under these same conditions.

Characterization of spontaneous revertants derived from Lyt⁻ strains MH-3 and MH-5. Spontaneous revertants of mutants MH-5 (MH-5R1 and MH-5R2) and MH-3 (MH-3R1 to MH-3R9) having both partially or fully restored phosphoglucomutase and autolytic amidase activities have previously been described (13). These were selected for their ability to grow on galactose as a carbon source. An additional Pgm⁺ revertant of MH-5, strain MH-5R6, was obtained in this study in a similar manner.

Each of the three revertants of MH-5 grew in MG and SGG media as motile, fully separated bacteria, having peritrichous flagella. The pattern of motility by each strain was similar to that of MH-1. The motile nature of the revertants was confirmed on NGA. Strains MH-5R1 and MH-5R2 were previously reported to contain near-normal levels of autolytic amidase (13), and this was found here to be true also for strain MH-5R6 (Fig. 7). Strain MH-5R6 was also observed to have fully restored β-*N*-acetylglucosaminidase activity (Fig. 8). The other two revertants were not tested for this autolysin.

None of the nine revertants of MH-3 exhibited motility when tested on semisolid Pen A, and seven of them (MH-3R2 and MH-3R4 to MH-3R9) neither exhibited motility nor formed flagella when grown in MG and SGG, even when the latter were supplemented by galactose. A typical revertant is shown in Fig. 5H. Strains MH-3R1 and MH-3R3 did not grow well in the minimal media and were not tested further.

The lytic activities of two of the MH-3 revertants, strains MH-3R2 and MH-3R4, are shown in Fig. 7 and 8. Both revertants contained only slightly higher levels of autolytic amidase and β-*N*-acetylglucosaminidase than mutant MH-3. Previously Forsberg and Rogers (13) also observed that amidase and phosphoglucomutase activities were only partially restored in MH-3R2 (by 25 and 31%, respectively), and similar results were obtained for revertant MH-3R1. The low lytic activity in these MH-3 revertants would probably explain their inability to form flagella. Further support for this is presented below.

To approach the problem from the opposite direction, a search was undertaken for spontaneous motility revertants of MH-3 and MH-5, with the expectation that such revertants would have restored phosphoglucomutase and lytic activities. One revertant of MH-3 (MH-3R10) and two of MH-5 (MH5-R4 and MH5-R5) were obtained. The phenotypic properties of the three revertants were nearly identical to those of the Pgm⁺ revertants of MH-5 described above. The three strains could utilize galactose as a carbon source, and each was found to contain wild-type levels of both amidase and β-*N*-acetylglucos-

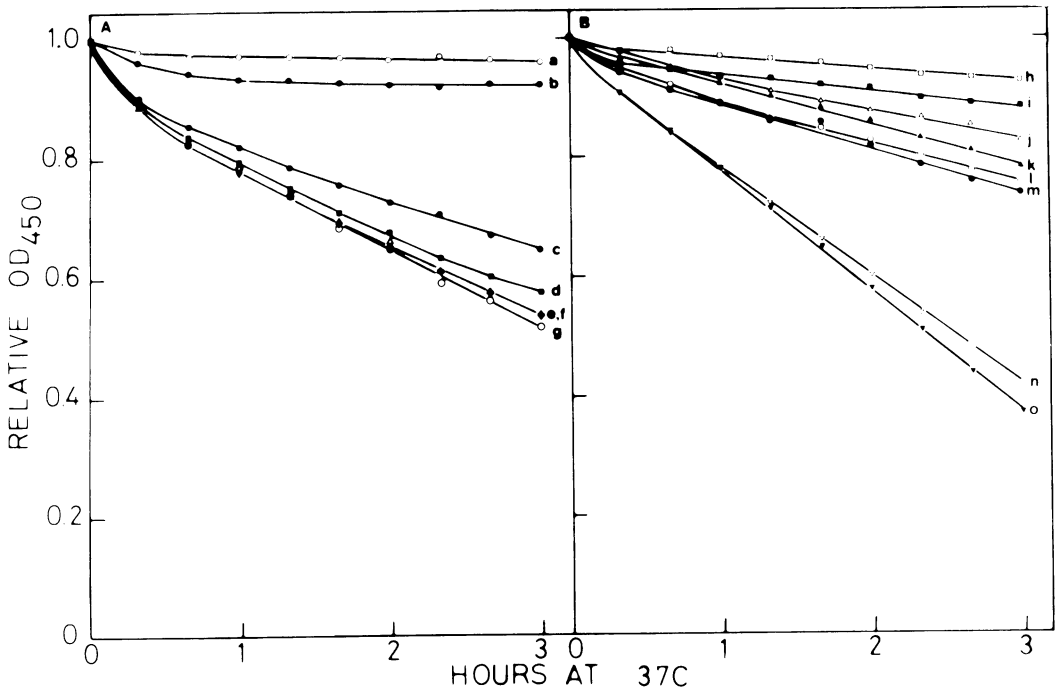

FIG. 7. *Lysis of SDS-treated cell walls of B. licheniformis 6346 (strain MH-1) at pH 9.5 in 0.025 M borate buffer containing 0.01 M $MgCl_2$, with crude LiCl extracts made from strains of B. licheniformis 6346 grown in SGG medium with (+gal) or without (−gal) added galactose. Curves and rough approximations of the wall lytic activities in units (U): (A) a, control, no added extract (○); b, MH-5, −gal (●, 0.31 U); c, MH-5, +gal (●, 1.83 U); d, MH-1, −gal (■, 2.31 U); e, MH-5R4, −gal (▼, 2.44 U); f, MH-5R6, −gal (△, 2.36 U); g, MH-5R5, −gal (○, 2.38 U). (B) h, MH-3, −gal (□, 0.19 U); i, MH-3, +gal (■, 0.31 U); j, MH-3R2, −gal (△, 0.79 U); k, MH-3R2, +gal (▲, 1.04 U); l, MH-3R4, −gal (○, 1.03 U); m, MH-3R4, +gal (●, 1.09 U); n, MH-3R10, −gal (▽, 2.79 U); o, MH-3R10, +gal (▼, 2.98 U).*

aminidase when grown in SGG (Fig. 7 and 8). In this medium, the cells formed peritrichous flagella and displayed the normal pattern of motility. Revertant MH-3R10 (Fig. 5I) is therefore quite unlike the partial Pgm⁺ revertants of MH-3 described above. The studies with this revertant provide additional support for the proposed autolysin-motility relationship in *B. licheniformis* 6346.

An additional type of motility revertant of MH-5, having somewhat different properties from those of the other revertants, was also isolated and characterized, (strain MH-5R3). In liquid and in semisolid minimal media containing glycerol as carbon source, the bacteria were motile and fully flagellated. Cell separation, however, was not as extensive as that measured for MH-1 (11.7 ± 9.5 cells per chain for MG-grown bacteria). Strain MH-5R3 could not grow on galactose as a sole carbon source, and was found to be Pgm⁻ (Table 3). The strain was also grossly deficient in autolytic amidase when grown in MG, but contained an increased level of β-*N*-acetylglucosaminidase as compared with MH-5 (Fig. 6).

It is known that the β-*N*-acetylglucosaminidases of *B. licheniformis* (13) and *B. subtilis* (9) are sensitive to proteolytic attack, and that protease-deficient mutants of these species can contain elevated levels of this autolysin. The proteolytic activity of MH-5R3 was screened on a skim-milk agar and was found to be reduced in comparison to MH-5.

Pooley et al. (H. Pooley, D. Belgrove, and C. Forsberg, First Int. Congr. Bacteriol., Jerusalem, **2**:90, 1973) described another protease-deficient partial "revertant" of MH-5 that has similar lytic and phosphoglucomutase properties to MH-5R3 (strain MH-5C). Further investigation has shown that this partial revertant, like MH-5R3, grows in liquid and semisolid glycerol minimal media as motile, fully flagellated and separated bacteria (1.7 ± 0.7 cells per chain in MG). Cells of MH-5C cultured in MG contained little autolytic amidase but normal levels of β-*N*-acetylglucosaminidase (Fig. 6). The level of the

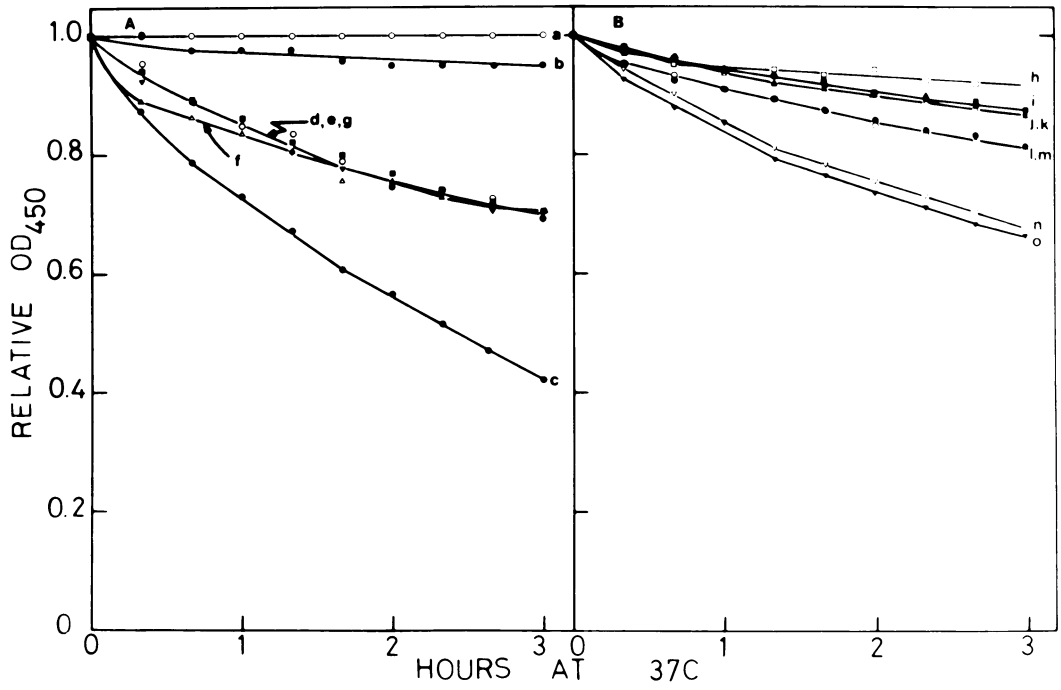

FIG. 8. *Lysis of SDS-treated cell walls of M. luteus at pH 5.6 in 0.05 M acetate buffer containing 0.01 M $MgCl_2$, with crude LiCl extracts made from strains of B. licheniformis 6346 grown in SGG with and without galactose. Symbols are as in Fig. 7. Curves and rough approximations of the wall lytic activities in units (U): (A) a, control, no added extract; b, MH-5, −gal (0.45 U); c, MH-5, +gal (3.49 U); d, MH-1, −gal (1.68 U); e, MH-5R4, −gal (1.99 U); f, MH-5R6, −gal (1.57 U); g, MH-5R5, +gal (1.77 U). (B) h, MH-3, −gal (0.25 U); i, MH-3, +gal (0.63 U); j, MH-3R2, −gal (1.06 U); k, MH-3R2, −gal (1.17 U); l, MH-3R4, −gal (0.96 U); m, MH-3R4, +gal (1.00 U); n, MH-3R10, −gal (2.42 U); o, MH-3R10, +gal (2.50 U).*

glycosidase was notably higher than that found in MH-5R3, and this might explain why daughter cell separation is more complete in MH-5C. Native walls prepared from both partial revertants grown in MG were found to bear the same lytic relationships at pH 5.6 and pH 9.5 as the LiCl extracts.

DISCUSSION

The work presented here strongly suggests that autolytic enzymes play an essential role in flagellar morphogenesis in species of *Bacillus*. This is based on the following observations. (i) Mutants of *B. subtilis* 168 (strains carrying the *lyt-1* or *lyt-2* mutation, and strain Nil5) and *B. licheniformis* 6346 (MH-3, MH-5) that are grossly deficient in both autolytic amidase and β-*N*-acetylglucosaminidase activities were found to lack motility and to be deficient in flagellar morphogenesis. (ii) Lyt^- transformants of *B. subtilis* 168 were previously constructed by congression ([12], including FJ3, FJ6, and FJ7). Each transformant was nonmotile, nonflagellated, and deficient in both autolysins. (iii) The phenotypes of two of the Lyt^- mutants investigated are conditional (12, 15). Under conditions permissive for synthesis of both autolysins (FJ3 grown at 17°C, and MH-5 grown with galactose), these strains were motile and flagellated. (iv) Spontaneous revertants derived from each of the poorly lytic mutants (with the exception of some partial revertants of MH-3 and MH-5) were peritrichously flagellated and normal in their patterns of motility, and each contained high levels of both autolytic amidase and β-*N*-acetylglucosaminidase. The partial Pgm^+ revertants of MH-3 (MH-3R1 to MH-3R9) were devoid of flagella and apparently contained only low levels of phosphoglucomutase and autolysins (Fig. 7 and 8; 13). The restoration of motility in two partial revertants of mutant MH-5 (strains MH-5C and MH-5R3) was correlated with increased β-*N*-acetylglucosaminidase activity in these protease-deficient strains. These revertants are also Pgm^-. It is therefore unlikely that the defect in flagellar morphogenesis in strain MH-5 results directly from the changed cell wall composition of this Pgm^- mutant, but

TABLE 3. *Phosphoglucomutase activities in strains of B. licheniformis 6346*

Strain	Phosphoglucomutase sp act[a]
MH-1	128.3
MH-5	<1.0
MH-5R3	<1.0

[a] Expressed as units × 10^3 per milligram of protein in the extracts, where 1 unit of enzyme activity is defined as the amount of enzyme utilizing 1 μmol of substrate per min.

rather from its deficiency in autolytic activity.

To explain how autolysins might be involved in flagellar morphogenesis, it is necessary to consider the structure of the flagellum and its relationship to the bacterial cell wall. The flagellum is a relatively complex organelle, consisting of three morphologically and chemically distinct parts: the filament, the hook, and the basal structure (24, 35). Of prime interest here is the basal structure, the component that traverses the cell wall and which is thought to function in the anchoring of the flagellum to the cell surface (24, 35). The basal structure is also probably involved in the assembly and functioning of the organelle. Electron microscopy has shown that the basal structure of *B. subtilis* consists of two rings or disks, designated M and S, that are mounted on a short, possibly hollow rod. The M ring appears to attach to the cytoplasmic membrane and the S ring to the inner surface of the cell wall. The short rod extends through the wall and appears to attach to the hook structure near the cell surface (24, 35). The basal structures of gram-negative bacteria are similar, but contain an additional pair of rings for binding to the more complex cell envelopes of these bacteria. Recent work with several bacteria including *B. subtilis* suggests that flagella elongate by the addition of identical polypeptide subunits (flagellin) to the distal tips of the growing filaments (24, 35). These monomers may be transported to the distal sites of assembly via a hollow channel running through the organelle, and the basal structure may play an active role in the transport process.

In line with all these observations, and with those made here with the poorly lytic mutants, I would like to propose as a working hypothesis that in flagellar morphogenesis a certain degree of localized peptidoglycan hydrolysis is required to allow for the assembly and insertion of the basal structures in the gram-positive cell wall. Since the basal structure appears to be required for flagellar synthesis and functioning, this model could explain the loss of motility and defective flagellar morphogenesis of the poorly lytic mutants. Presumably in the case of mutant MH-5 grown in SGG, where many of the cells are flagellated, the basal structures are not set properly in the wall, accounting for the loss of normal motility in the strain. Hollow pores or channels through which flagella are thought to pass have been detected in the cell walls of *Clostridium sporogenes* (5) and, recently, in *Aquaspirillum serpens* (R. G. E. Murray, Abstr. Annu. Meet. Can. Soc. Microbiol. 1978, M1). Similar structures for fimbriae have been reported to occur in the peptidoglycan layer of certain gram-negative species (20). Such pores could possibly be formed by the action of autolytic enzymes, in conjunction with the synthesis of the appendages.

There have been several other reports in the recent literature suggesting a close association between the bacterial cell surface and flagellar morphogenesis. Studies with several gram-negative species have indicated that flagellar synthesis is dependent on either the presence or concurrent synthesis of an intact cell envelope (29, 40). Other work has shown that loss of ability to synthesize flagella can be brought about by mutations that alter the structure and composition of the cell envelope. This, for instance, has been observed with some deep rough mutants of *Salmonella typhimurium* (2, 25) and *galU* mutants of *E. coli* (26). In *E. coli*, several polypeptides concerned with flagellar rotation and chemotaxis are located in the cytoplasmic membrane (35).

In view of the close relationship between the cell surface and flagellar formation, and the active role played by autolysins in cell wall metabolism, it is perhaps not too surprising that the poorly lytic mutants described here are deficient in flagellation. Several nonmotile, pleiotropic mutants of *B. subtilis* 168 were recently described which lack flagella (but synthesize flagellin) and which also are partially deficient in autolytic enzyme activity (3, 37, 42). These mutants are quite unlike Lyt⁻ strains FJ3 and FJ6 (12) in that they are hyperproducers of several extracellular enzymes (α-amylase, neutral and alkaline proteases, and levansucrase), are poorly or not at all transformable, and form only short chains of unseparated bacteria in rich growth media (indicating that they are more lytic than strains FJ3 and FJ6). Because of the pleiotropic nature of these mutants, it is not known if their lack of flagella is a direct consequence of their decreased lytic activity, and several possible explanations for their phenotypes have been suggested (3, 37).

Strain Nil5 appears to have a closely similar phenotype to that of mutants FJ3 and FJ6, in

terms of autolysin production, chain formation, and loss of motility and flagellation. Pooley (31) previously reported that Nil5 has a significantly reduced rate of peptidoglycan turnover during growth, and similar observations have been made with strains FJ3 and FJ6 (R. S. Buxton, J. E. Fein, and H. J. Rogers, unpublished data). Strain Nil5 was derived from *B. subtilis* 168 Trp Thy⁻ after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis (31), and was selected for its failure to lyse upon thymine starvation (due to induction of phage PBSX). In view of the choice of mutagen (17) and the isolation here of a spontaneous, Lyt⁺ PBSX⁻ revertant of Nil5 (FJ60), it is likely that Nil5 is a multiple mutant and that the lytic and PBSX defects in it are not directly related. In support of this, PBSX induction was found to be normal in Lyt⁻ strains FJ3 and FJ6 (12).

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