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Autolytic Enzyme-Deficient Mutants of *Bacillus subtilis* 168

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Mutants of *Bacillus subtilis* strain 168 have been isolated that are at least 90 to 95% deficient in the autolytic enzymes *N*-acetylmuramyl-L-alanine amidase and endo-β-*N*-acetylglucosaminidase. These mutants grow at normal rates as very long chains of unseparated cells. The length of the chains is directly related to the growth rates. They are nonmotile and have no flagella, but otherwise appear to have normal cell morphology. Their walls are fully susceptible to enzymes formed by the wild type and have the same chemical composition as the latter. Cell wall preparations from the mutants lyse at about 10% of the rate of those from the isogenic wild type, with the correspondingly small liberation of both the amino groups of alanine at pH 8.0 and of reducing groups at pH 5.6. Likewise, *Micrococcus luteus* walls at pH 5.6 and *B. subtilis* walls at pH 8 are lysed only very slowly by LiCl extracts made from the mutants as compared with rates obtained with wild-type extracts. Thus, the activity of both autolytic enzymes in the mutants is depressed. The frequencies of transformation, the isolation of revertants, and observations with a temperature-sensitive mutant all point to the likelihood that the pleiotropic, phenotypic properties of the strains are due to a single mutation. The mutants did not produce more protease or amylase than did the wild type. They sporulate and the spores germinate normally. The addition of antibiotics to exponentially growing cultures prevents wall synthesis but leads to less lysis than is obtained with the wild type. The bacteriophage PBSX can be induced in the mutants by treatment with mitomycin C.

It could be predicted that the ability of bacteria to autolyze by enzymatic degradation of their walls might be affected by changes in the substrate, i.e., the wall, or by deficient production of the relevant enzymes. The latter might be a direct effect, the result of the failure of a proenzyme activator system or the result of the hyperproduction of an autolysin inhibitor. Among the autolytic enzyme-deficient microorganisms so far described, the involvement of several of these possible factors can be discerned. For example, pneumococci grown in the presence of ethanolamine instead of choline and mutants of *Bacillus licheniformis* deficient in phosphoglucomutase have walls that are resistant to their own major autolytic enzyme, but also produce less or changed enzyme (18, 19, 56, 60). Other mutants of pneumococci (30) may either have a deficiency in autolytic enzyme formation or be hyperproducers of lipoteichoic acid, an inhibitor of autolytic enzymes (9, 26, 59). Mutants of *Streptococcus faecalis* have been isolated (38) that had walls sensitive to added autolysins but were partially defective in this activity and which might have had a defective proenzyme. Mutants of *B. subtilis* with a temperature-sensitive autolytic amidase have been claimed to occur in cultures repeatedly treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (15). Nonmotile, pleiotropic, partially autolytic enzyme-deficient mutants of this organism that are also hyperproducers of amylase and protease have been isolated and carefully studied genetically (6, 62).

The aim of the present work was to isolate mutants from *B. subtilis* 168 that are grossly deficient in the production of autolytic enzymes, but with walls that are unchanged both in their chemistry and in their susceptibility to the autolytic enzymes produced by the wild type. These were then used to examine the claim (14, 17) and the counterclaim (18, 19) that deficiency in autolytic enzymes leads to reduced growth rates. Further evidence is also required for the correlation between deficiency in autolytic enzymes, cell separation (12, 18, 19, 38, 41, 56), transformability (1, 2, 39, 48, 64), and tolerance to antibiotics (40, 42, 43, 57, 58). The mutants studied in this work have been isolated after minimal mutagenesis and without the use of deliberately selective conditions. Evidence is presented that a single mutation is involved.

MATERIALS AND METHODS

Microorganisms. The mutants of *B. subtilis* strain 168 that were used or isolated are listed in

Table 1 along with their genotypes, phenotypes, and derivations or sources. *Micrococcus luteus* (*lysodeikticus*) NCTC 2665 and *B. subtilis* strain W23 were also used. All cultures were kept in the freeze-dried state. Phage 29 was obtained from R. F. Rosenberger of this department.

Media. The following media were used: Penassay broth (Pen B); Spizizen salts-glucose medium (51) supplemented by $MnSO_4 \cdot 4H_2O$ (0.01 μg/ml) and, where indicated, by sodium L-glutamate (0.3%) (SMM); and a salts-glucose medium (MSM) originally designed to allow the maximum separation of bacterial cells during growth (45). This was supplemented by L-arginine (0.2%), where indicated, and a casein-yeast hydrolysate medium (28) (CH). Penassay broth solidified with 0.5% agar was used to isolate the revertants. Amino acids (20 μg/ml) were added to the minimal media when necessary to meet the auxotrophic requirements of the various mutants. With inocula from nutrient agar cultures, L-alanine (100 μg/ml) was added to encourage the germination of any spores.

Cultural conditions and measurements of growth. Liquid cultures were grown in conical flasks containing 20% of their volume as medium and were inoculated from single colonies from agar plates. They were incubated at 35°C, unless otherwise stated, and shaken mechanically to ensure adequate aeration. At intervals samples were taken into 0.5-ml curvettes, and the extinction at 675 nm was measured, using a Unicam SP-600. Deviations from Beers Law were corrected as by Toennies and Gallant (55).

Preparations of cell walls containing autolysin (native walls). Bacteria from exponential-phase cultures (absorbancy at 675 nm [A_{675}] of 1.0 to 1.5) growing in Pen B medium were deposited by centrifuging (16,000 × *g* for 5 min) at 4°C. All the subsequent treatments were at 0 to 4°C. The bacteria were suspended in a small volume of TL buffer, pH 8.0 [0.05 M tris(hydroxymethyl)aminomethane-hydrochloride containing 0.41 M LiCl; final ionic strength, 0.07], and disrupted by ultrasonic oscillation (three 1.0-min bursts) of a Branson sonifier (Dawe Instruments, London) at maximum power, while keeping the vessel containing the suspension immersed in an ice-water mixture. Undisrupted bacteria were removed by slow-speed centrifugation (1,000 × *g* for 5 min). The walls in the resultant supernatant fluid were deposited at 27,000 × *g* for 5 min. They were washed three times with TL buffer and resuspended in TL buffer containing 0.01 M $MgCl_2$ to approximately 1 mg (dry weight) per ml. Some batches indicated in the text were made with other buffers. Lithium chloride was added to the buffers to adjust the ionic strength to a constant value of 0.07 because this salt was also present in the autolysin-containing extracts (see below).

Preparation of SDS-treated walls. The strains were grown to the middle of the exponential phase in SMM medium supplemented by sodium L-glutamate, in 50- or 200-liter fermentors using forced aeration. The cultures were rapidly cooled by passing cold water through the jackets of the fermentors, and the bacteria were deposited with a Sharples continuous centrifuge. The resulting cell paste was taken out of the rotor and suspended to 12 to 14 mg (dry weight) per ml in 4% (wt/vol) sodium dodecyl sulfate (SDS). Rapid and even suspension was achieved by using a Silverston homogenizer, followed by stirring for at least 2 h at room temperature. The suspension was then passed through a grade 2 sintered-glass filter and disrupted in a cell breaker (50) at approximately 1.5×10^4 lb/in^2. The walls and undisrupted cells were deposited from the mixture by centrifugation and were suspended in 4% (wt/vol) SDS. The temperature of the suspension was raised to 100°C for 5 to 10 min. They were then deposited again by centrifugation and washed successively, once with 4% SDS, once with water, three times with 1 M NaCl, and then again four times with water. After each centrifugation, the wall layer in the deposit was mechanically separated from the underlying whole cells and other contaminating material. The walls were finally freeze-dried and, when necessary, coupled with Procion brilliant red (18).

Walls from *M. luteus* were prepared from exponential-phase cultures grown in CH medium by the same method, except that the cells were disrupted by 3 min of treatment in a Braun homogenizer.

Isolation of autolytic enzyme-deficient mutants. When the method was designed, two considerations were borne in mind: (i) *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was avoided as a mutagen due to its

TABLE 1. *B. subtilis* strains

Strain	Genotype	Phenotype	Origin
168	*trpC2*	Trp$^-$	J. Spizizen
MB21	*metC3 leu-8 tal-1*	Met$^-$ Leu$^-$	P. J. Piggot (27)
BD112	*cysA14*	Cys$^-$	D. Dubnau
FJ1	*trpC2 lyt-1*	Trp$^-$ Lyt$^-$-ts	Derived by ethyl methane sulfonate mutagenesis of 168
FJ2	*trpC2 lyt-2*	Trp$^-$ Lyt$^-$	
FJ3	*metC3 lyt-1*	Met$^-$ Lyt$^-$-ts	Derived by transformation of MB21 by DNA from FJ1 and FJ2
FJ6	*metC3 lyt-2*	Met$^-$ Lyt$^-$	
FJ7	*metC3 lyt-2*	Met$^-$ Lyt$^-$	
FJ8	*metC3*	Met$^-$ Lyt$^+$	Derived by transformants of MB21 with DNA from FJ2
W23		Prototrophic	Stock strain
Ni15	*trpC2 thyA thyB*	Trp$^-$ Thy$^-$ PBSX$^-$ Lyt$^-$	D. Karamata

notorious ability to cause multiple mutations (24); (ii) it was desirable to obtain temperature-conditional mutants. Exponential cultures of *B. subtilis* 168 *trpC2* grown in Pen B medium at 35°C (A_{675} = 1.2) were treated with ethyl methane sulfonate (0.2 ml/10 ml of culture) for 60 min at 30°C with mechanical agitation. The bacteria were harvested by centrifugation, washed twice, and suspended in SMM containing L-tryptophan (20 μg/ml). These cultures were divided into several flasks and incubated overnight at 30°C with shaking. In the morning the cells were transferred to fresh medium (starting A_{675} = 0.07), and incubation was continued at 30°C until the cultures reached early exponential phase (A_{675} = 0.17). They were then transferred to a 45°C shaking water bath for 70 min. To enrich for autolytic enzyme-deficient mutants, methicillin (25 μg/ml) was added in the supposition that this antibiotic might be bacteriostatic rather than bactericidal to such mutants (18, 57, 58). In the presence of the antibiotic, the optical density of the cultures dropped 71% (i.e., from 0.43 to 0.125) in 60 min. The cells were then cooled on ice, harvested by centrifugation, washed twice and resuspended in SMM medium containing L-tryptophan, and plated onto similar medium solidified with 2% (wt/vol) agar. After 72 h at 30°C, bacteria from single colonies were transferred to agar plates containing Procion brilliant red-stained walls (18) isolated from *B. subtilis* 168 *trpC2*. To conserve the stained walls, sandwich plates were used, consisting of a 15-ml base layer of solidified SMM containing L-tryptophan (2% agar) and an overlay of 7 ml of the same medium but solidified with 0.9% agar and containing 1.1 mg of the Procion brilliant red coupled walls. The plates were incubated for 2 to 4 days at 30°C, followed by 1 to 3 h at 45°C. They were then incubated anaerobically in a jar for 24 to 36 h at 45°C. Colonies failing to form zones of clearing around them were selected and purified three times. Colonies were always selected from plates originally inoculated from different liquid cultures. By this procedure, strain FJ2 was isolated.

The procedure used to isolate strain FJ1 was basically as above, except that after methicillin treatment (15 min at 45°C in Pen B medium), the cells were subjected to a second enrichment procedure based on autolysis under semi-anaerobic conditions. The methicillin-treated cells were harvested at 20°C, washed, and resuspended (approximately 0.2 mg of cell dry weight per ml) in 0.05 M sodium carbonate buffer (pH 9.5). This suspension was incubated at 45°C in sealed tubes filled with culture for 30 min, by which time autolysis had caused a drop in turbidity of approximately 40%. The cells were then harvested, washed once, resuspended in SMM medium containing L-tryptophan, and plated directly onto sandwich plates containing Procion brilliant red coupled walls (18). The treatment of the plates and the isolation of strains were as described above for the isolation of FJ2.

Materials. Procion brilliant red was purchased from R. A. Lamb and Co., Middlesex, England; ethyl methane sulfonate was from Sigma Chemical Co., St. Louis, Mo.; and Hide Powder Azure was from Calbiochem, San Diego, Calif. All other chemicals were of reagent grade as obtained from commercial sources.

Lysis of whole cells. Cultures growing exponentially in Pen B medium at 45°C were centrifuged and resuspended in ice-cold 0.05 M carbonate buffer, pH 9.5. Narrow, optically matched test tubes were completely filled with the buffered suspensions and sealed. They were incubated at 45°C. The extinction values at 675 nm were measured at intervals and were corrected as by Toennies and Gallant (55).

Lysis of wall preparations. Native walls were suspended at about 1 mg/ml in either ice-cold TL buffer, pH 8.0, containing 0.01 M $MgCl_2$ or in 0.1 M acetate buffer, pH 5.6, containing 0.01 M $MgCl_2$. The suspensions (4 ml) were measured into optically matched tubes. Lysis was started by transferring the tubes to 37°C, and they were then shaken mechanically during incubation to keep the wall preparation suspended. At intervals the optical densities were read and samples were taken from the pH 8 tubes for the determination of the free $-NH_2$ groups and from the pH 5.6 tubes for the determination of the liberation of reducing groups. A unit of lytic activity was taken as that which lowered the A_{450} of the suspensions by 0.001/min during the period of approximately linear decrease.

Preparation and activity of LiCl extracts. The method used for the preparation of LiCl extracts was essentially that of Brown (7). Bacteria from the midexponential phase of growing cultures in Pen B medium were washed in water at 0°C and dried from the frozen state or, if they were to be used immediately, they were washed and prepared as a paste in cold TL buffer. The cells in either case were extracted with 5 M LiCl at 0°C for 30 to 60 min, after which time the mixture was centrifuged. Exactly 200 mg (dry weight) of freeze-dried cells of LiCl per ml was used, but with fresh cells around 400 mg/ml was used. The supernatant fluids containing autolysin were partially purified by adsorption and elution from SDS-treated cell walls (7) These eluates were then diluted with water and stored at 0°C. The lytic activities in these extracts were tested at 37°C by adding 0.12 ml of the extracts to 3.87 ml of (i) suspensions (1 mg/ml) of SDS-treated walls from the mutants and wild-type *B. subtilis* in 0.05 M tris(hydroxymethyl)aminomethane buffer, pH 8.0, containing 0.01 M $MgCl_2$ and (ii) suspensions (0.5 mg/ml) of *M. luteus* walls in 0.05 M sodium acetate buffer, pH 5.6, also containing 0.01 M $MgCl_2$. All the wall suspensions were sonically treated for 5 s at 0°C to obtain even suspensions before the addition of the extracts. Lysin was measured by the diminution of extinction values as above. Methods (i) and (ii) favor, respectively, the action of the *N*-acetylmuramyl-L-alanine amidase and β-*N*-acetylglucosaminidase (16).

Determination of numbers of cells in chains. Samples of exponential cultures growing in minimal media were gently mixed with 5% formalin to fix them and then stained with 0.01% Victoria blue to show the septa clearly (M. G. Sargent, unpublished data). The fixed, unstained cells were also photographed with a phase microscope at a magnification

of ×1,500. Bacteria with active autolysin had to be heat fixed before photography to immobilize them.

Enzymatic and chemical assays. Amino acids and amino sugars were determined after acid hydrolysis of wall preparations with a Beckman Spinco automatic amino acid analyzer. Hydrolysis was either with 6 N HCl for 16 h or 4 N HCl for 4 h at 100°C, respectively, for amino acids or amino sugars. Hydrolysis in preparation for phosphorus, glucose, and glycerol determinations was with 2 N HCl for 2 h at 100°C. Phosphorus was then determined by the method of Ames and Dubin (3), glucose was determined with hexokinase and glucose-6-phosphate dehydrogenase (52), and glycerol was determined with glycerol dehydrogenase after digestion with alkaline phosphatase (21). Phosphoglucomutase in disrupted bacteria was estimated by the method of Forsberg et al. (21). Protein was determined by the method of Lowry et al. (33), using bovine plasma albumin as standard. Hexuronic acid estimations were as by Dische (11) on unhydrolyzed wall preparations. Liberated L-alanyl NH_2 groups were measured by the method of Forsberg and Ward (20). Reducing groups were measured by the method of Thompson and Shockman (54).

Transformation and construction of isogenic strains. The recipient strain MB21 was grown to competence (4) and transformed by saturating concentrations of deoxyribonucleic acid (DNA) (5 μg/ml) isolated by the method of Marmur (34). Donor DNA concentrations were measured by the method of Giles and Myers (22). In constructing the isogenic wild-type strains, low concentrations (0.05 μg/ml) of donor DNA were used.

Protease formation. The method of Dancer and Mandelstam (10) was used for protease formation, which entails incubation of the bacteria in a medium inducing sporulation and measurement of the hydrolysis of Remazol brilliant blue hide by the supernatant fluids from these cultures. The strains were also grown on skimmed milk agar (5% [wt/vol] skimmed milk [Difco] solidified with 1.2% [wt/vol] agar) and a casein agar medium (25). The zones of proteolysis were measured after various times of incubation of the plates at 35°C.

Amylase formation. The strains were grown at 35°C for up to 96 h on starch peptone agar. The plates were stained with KI-I_2 solution and fixed (23). The diameter of the unstained zones on separate sets of plates was measured after 24, 48, and 96 h of incubation.

Preparation of spores and their germination. Germination was studied quantitatively by using spores prepared from 48 h of growth on Schaeffer nutrient agar (47). The growth was scraped from the agar surface, washed twice with cold water, and suspended in 0.85% NaCl containing 200 μg of lysozyme per ml. The suspension was incubated for 30 min at 20°C and centrifuged, and the cells were suspended in 1% (wt/vol) SDS. It was again kept for 30 min at 20°C and centrifuged, and the spores were washed six times with water at 0 to 4°C. Before germination, the suspension (about 10^9 spores/ml) was heated at 80°C for 15 min to kill any remaining vegetative cells and to "activate" the spores. Germination of these spores was studied by following the fall in A_{580} at 37°C while they were suspended in Pen B medium (61). The suspension was first adjusted to an A_{580} of 0.400 before incubation.

RESULTS

Lysis of whole cells and walls. Four mutants showing very much reduced or no lytic zones on the Procion agar plates were isolated. Of these, two strains, FJ1 and FJ2, were selected for detailed study. The rate of lysis of cell suspensions incubated at pH 9.5 under semi-anaerobic conditions was much reduced (Fig. 1). Native walls prepared from these mutants likewise lysed at pH 8.6 much more slowly, the rate of reduction of A_{450} being about 5% of that of walls from the parent strain 168 *trpC*. Liberation of the free amino groups of alanine, presumably arising from the hydrolysis of the bond between the *N*-acetylmuramyl residues and the L-alanine of the mucopeptide of the walls, was likewise reduced in mutant FJ2 by about 95 to 96% (Fig. 2). After it had been established that the mutants isolated were in fact poorly lytic, their DNA was isolated and used to transform strain MB21. Saturating doses of DNA from both FJ1 and FJ2 were used in separate experiments, and L-leucine-independent transformants were selected on SMM medium containing L-glutamate, L-methionine, and L-tryptophan. Transformants were streaked on the same medium and colonies from these plates were transferred to nutrient agar. Tryptophan-dependent and methionine-independent colonies were then sought by growing on SMM containing the

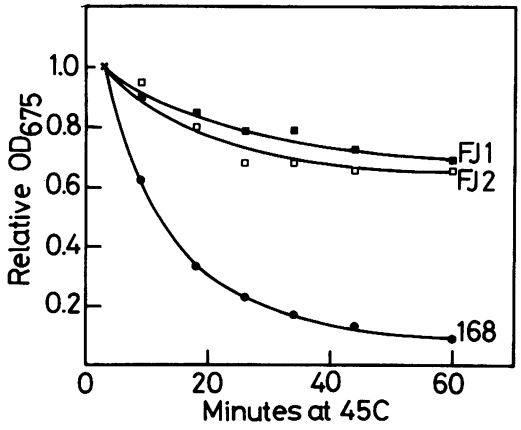

FIG. 1. *Autolysis at pH 9.5 of whole bacteria from exponential-phase cultures of B. subtilis 168 and of the autolytic enzyme-deficient mutant strains FJ1 and FJ2. Symbols: ●, B. subtilis; ■, FJ1; □, FJ2. OD_{675}, Optical density at 675 nm.*

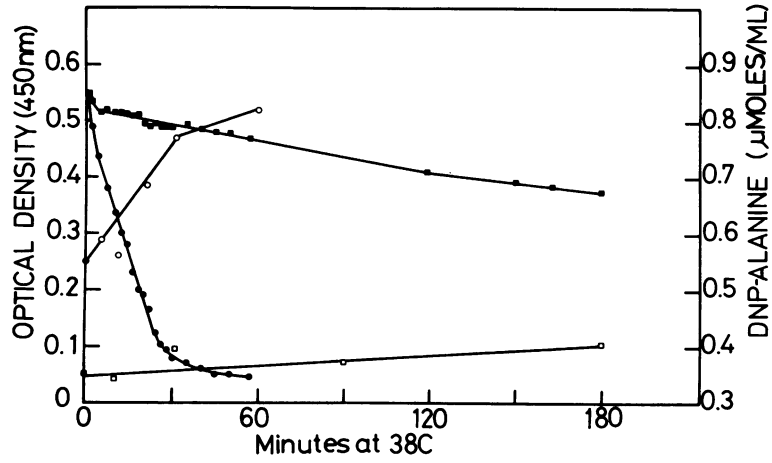

FIG. 2. *Autolysis of native walls isolated from B. subtilis 168 (●, ○) and from the mutant strain FJ2 (■, □) at pH 8.6 in 0.012 M borate buffer containing 0.075 M LiCl (BL buffer; final ionic strength, 0.1). Native walls were prepared using cold BL buffer and were suspended to approximately 1 mg/ml. Closed symbols, decrease in optical density; open symbols, increase in N-terminal alanine. The rates calculated from the initial slopes of the curves were 16.7 U or 7.0 nmol/min per ml and 0.8 U or 0.3 nmol/min per ml for B. subtilis 168 and strain FJ2, respectively.*

appropriate amino acids. Autolytic enzyme deficiency was examined on Procion red wall agar containing methionine and tryptophan. The results of these experiments are summarized in Table 2. An isogenic wild type with respect to autolytic activity was constructed by using low doses of strain FJ2 donor DNA and selecting for leucine independence. This strain was called FJ8. The rates of lysis of native walls from strain MB21 were less rapid than those of strain 168 *trpC2*. Nevertheless, a comparison of the rates of lysis and liberation of free amino groups by native walls of FJ3, 6, and 8 shows that the slow relative rates shown by those isolated from the original mutants were preserved in the transformants (Fig. 3). The rates measured by reduction in A_{450} for walls from FJ3 and FJ6 were, respectively, 16 or 12% of those from the isogenic wild-type FJ8, and the rates for the liberation of amino groups of alanine were 9.5 and 7.9%. It would thus appear that FJ3 and FJ6 expressed greatly reduced levels of activity of the autolytic amidase. Also present in the walls of *B. subtilis* is an endo-β-*N*-acetylglucosaminidase with a pH optimum of between pH 5.0 and 6.0 (8, 16). Figure 4 shows the rates of reduction of A_{450} of suspensions of walls incubated at pH 5.6, together with results for the liberation of reducing groups. It will be seen that these activities are also reduced in the walls from the mutant strain to approximately the same extent as are the amidase activities. LiCl (5 M) extracts were then prepared from FJ3, 6, and 8 and allowed to act upon SDS-treated walls prepared from FJ8 and *M. luteus*. The amidase and the β-*N*-acetylglucosaminidase activities of the extracts were in the same relationship as were the activities using native walls.

TABLE 2. *Results for the transformation of B. subtilis MB21 by congression*[a]

Source of donor DNA	No. of *leu*$^+$ transformants selected	Proportion of transformants (%)		
		lyt$^-$	*met*$^+$	*trp*$^-$
FJ1	173	4.6	2.3	4.6
FJ2	156	4.5	0[b]	0.6

[a] Transformation was performed using saturating doses of DNA, and *leu*$^+$ transformants were selected. Under these conditions some 5% of the selected transformants would also be expected to be transformed for unlinked markers (i.e., by congression).
[b] No colonies seen.

Chemistry of the walls. Analysis of acid hydrolysates of the walls (Table 3) shows that both mutant strains FJ3 and FJ6 and the isogenic wild-type strain (FJ8) have closely similar compositions, which in turn are similar to other published results for strain 168 (29). The teichoic acid content of the walls of all three strains is somewhat higher than that previously found for strain 168. The only potentially important departure, however, from the previous published results is the lower figure for the total alanine content. It would appear that this is due to the loss of the ester D-alanine

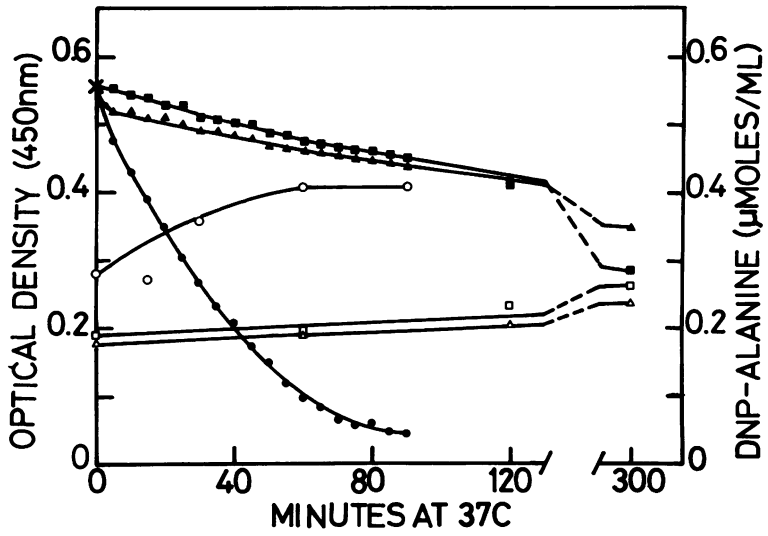

FIG. 3. *Comparative autolysis at pH 8 of native walls isolated from the transformant strains FJ3 (■, □) and FJ6 (▲, △) and from the isogenic wild-type FJ8 (●, ○) walls suspended to 1 mg/ml in 0.04 M HEPES(N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer containing 0.037 M LiCl (HL buffer) and 0.01 M $MgCl_2$ (final ionic strength, 0.1). Native walls were prepared using HL buffer. Ethylenediaminetetraacetic acid (1 mM) was added to the buffer used for cell breakage and for the first two washings of the walls. Closed symbols, decrease in optical density; open symbols, increase in N-terminal alanine. The rates of FJ3, FJ6 and FJ8 were 1.4 U or 0.24 nmol/min per ml, 1.3 U or 0.20 nmol/min per ml, and 8.7 U or 2.53 nmol/min per ml, respectively.*

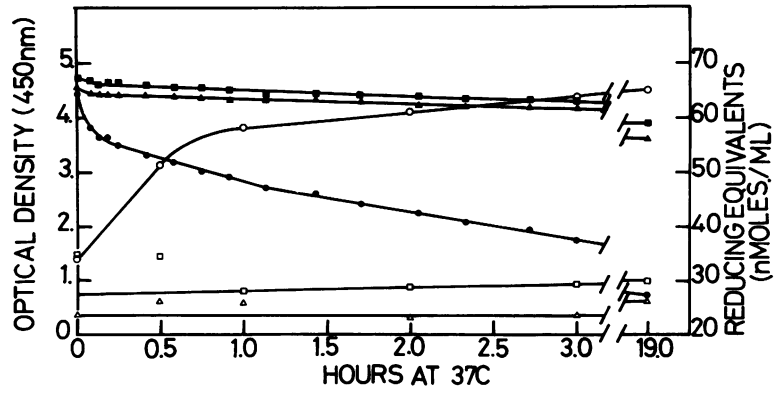

FIG. 4. *Comparative autolysis of native walls of strains FJ3 (■, □), FJ6 (▲, △), and FJ8 (●, ○) at pH 5.6 in 0.1 M sodium acetate buffer containing 0.01 M $MgCl_2$ (final ionic strength, 0.12). Native walls were prepared from cells disrupted in and washed with 0.1 M sodium acetate buffer, pH 5.6, and then resuspended in buffer containing 0.01 M $MgCl_2$ to approximately 6.7 mg (dry weight) per ml for reducing-power assay or to 0.67 mg (dry weight) per ml for turbidity measurements. Closed symbols, decrease in optical density; open symbols, increase in reducing power. The optical density values were corrected by a factor of 10.*

associated with the teichoic acid fraction of the walls rather than to a low peptidoglycan alanine content. No alanine was removed by treating the walls with 0.1 M NH_4OH, which hydrolyzes the labile ester bond. The reason for the loss or absence of the ester alanine is not known. It would seem likely that the composition of the walls of both mutants is closely similar to, if not identical with, the isogenic wild type in their content of peptidoglycan and teichoic acids.

When subjected to growth under phosphate-limiting conditions, the autolytic enzyme-deficient phosphoglucomutase mutants of *B. li-*

cheniformis, as well as similar mutants of *B. subtilis* and uridine diphosphate-glucose pyrophosphorylase-deficient mutants of both organisms (Forsberg and Rogers, unpublished data), failed to form teichuronic acid, produced walls consisting almost entirely of naked peptidoglycan, and were disturbed in morphology so that they resembled the *rod* mutants of *B. subtilis* (21). Limiting the growth of FJ6 by phosphate supply in the chemostat had none of these consequences. The individual bacteria had a rod shape, and the walls contained teichuronic acid. The chains of unseparated bacteria were not as long as in the exponential batch cultures (see below), but this might be explained either by physical breaking of the chains due to the high-speed stirring of the chemostat culture necessary for adequate aeration or to the slow growth rate.

Susceptibility of the walls from mutant strains to wild-type lysin. LiCl extracts were made from the isogenic wild-type strain and allowed to act upon the SDS-treated walls from the mutant strains, from FJ8 itself, and from strain 168 *trpC*. The rates of loss of turbidity by the suspensions were identical, showing that the walls from the mutant strains were fully susceptible to the enzyme from the wild type.

Growth rates and chain formation by the mutant strains. The growth rates of the mutants and of the isogenic wild type were very similar in the five media tested (Table 4). In some, the mutant may have grown very slightly slower although the greatest difference in doubling time observed was only 15%. Great differences were found between the numbers of unseparated but septated cells in the mutants growing at 35°C as compared with the wild type also growing at the same temperature (Table 5). There were also considerable differences between the lengths of the chains according to the growth medium, varying with the mutants from about 13 cells/chain in SMM medium to 200 cells in Pen B medium (Fig. 5). When the separation times (36, 46) for the cells were calculated (Table 6), the effects between the different media were much less striking, varying from about 160 min for Pen B medium to 380 min for MSM medium plus L-arginine. Inasmuch as cell separation in these mutants can be regarded as due to a balance between growth rate and the action of the residual autolytic

TABLE 4. *Growth rates of autolytic enzyme-deficient mutants and the isogenic wild type*

Medium	Growth temp (°C)	Doubling time (min)		
		Strain FJ3[a]	Strain FJ6[a]	Strain FJ8[b]
Pen B	17	178.2	205.0	179.2
Pen B	35	20.7	22.2	19.3
SMM	35	64.6	65.4	65.7
SMM + L-glutamate	35	53.2	51.6	50.4
MSM	35	59.8	59.4	58.6
MSM + L-arginine	35	51.5	50.7	51.0

[a] Lyt⁻ mutant.
[b] Isogenic wild type.

TABLE 3. *Chemistry of SDS-treated cell walls from the autolytic enzyme-deficient strains (FJ3 and FJ6) and the isogenic wild type (FJ8)*[a]

Components	Strain FJ8		Strain FJ3		Strain FJ6	
	μmol/100 mg	MR[b]	μmol/100 mg	MR	μmol/100 mg	MR
Muramic acid	23.3	0.73	24.0	0.63	25.6	0.64
Muramic acid + muramic acid phosphate	25.7	0.81	26.7	0.70	27.9	0.70
Glucosamine	36.6	1.15	35.9	0.94	39.2	0.99
Galactosamine	43.5	1.37	36.3	0.95	38.6	0.97
Glutamic acid	31.8	1	38.3	1	39.7	1
Alanine	53.9	1.69	59.9	1.56	68.7	1.73
2,6-Diaminopimelic acid	37.5	1.18	41.6	1.09	44.3	1.12
Glycerol	127.8	4.02	125.6	3.28	126.7	3.19
Glucose	103.9	3.27	104.7	2.73	104.1	2.62
PO_4^{2-}	173.6	5.49	171.7	4.48	172.5	4.35
Glycerol + galactosamine	171.3	5.39	161.9	4.23	165.3	4.16
Alanine after removal of ester alanine		1.69		1.65		1.6

[a] The bacteria were grown at 35°C in SMM medium supplemented with glutamic acid plus L-methionine (20 μg/ml). The cultures were harvested during the exponential phase of growth.
[b] MR, Molar ratio compared with D-glutamic acid.

TABLE 5. *Lengths of chains of unseparated but septated cells in exponentially growing cultures of the autolytic enzyme-deficient mutants*

Medium	Growth temp (°C)	No. of cells/chain		
		Strain FJ3[a]	Strain FJ6[a]	Strain FJ8[b]
Pen B	17	4.6 (9.5)[c] E	124.3 (49.3) B	2.4 (1.3) C
Pen B	35	201.1 (85.2) A	198.4 (69.5) A	6.1 (11.7) C
SMM	35	13.8 (8.5)[d] C	12.9 (7.1)[d] C	1.5 (0.5) C
SMM + L-glutamate	35	36.3 (22.1) C	31.6 (18.8) C	2.2 (1.3) C
MSM	35	71.2 (41.5)[d] B	86.6 (50.1)[d] B	1.9 (1.2) C
MSM + L-arginine	35	173.4 (97.9) A	189.9 (90.4) A	3.8 (6.0) D

[a] Lyt⁻ mutant.
[b] Isogenic wild type.
[c] The numbers in parentheses are the standard deviations for the mean results. The number of chains counted is indicated by the subscript letters where A = 25, B = 50, C = 100, D = 200, and E = 250.
[d] Some (about 1%) of the cells contained spores.

enzymes produced, the variation in lengths of the chains is more likely to be due to the differences between growth rates than between degrees of enzyme activity. It was noticed that in media in which long chains were formed, a very high proportion (at least 40 to 80%) of the chains, occasional individual, phase-light, apken cell wall fragments. Moreover, within the chains, occasional, individual, phase-light apparently empty cells were present (Fig. 5). Empty cells have been seen (49) in chains of *S. faecalis* growing in the presence of the culture fluid from overnight cultures of the organism. It was suggested (49) that chain breakage was taking place as the result of lysis of individual cells by enzymes in the culture fluid rather than by true cell separation. Whether the phase-light cells in the chains of the present mutants arise from the action of their own residual autolytic enzyme or by some other means is unknown. They may represent points of weakness and account for the wall fragments at the ends of the chains and for chain breakage.

One important result emerged from studying the effects of temperature on the lengths of the chains. As will be seen (Table 5, Fig. 5), when FJ3 was grown at 17°C instead of at 35°C, the average lengths of the chains were not very different from those of the wild type. No similar effect was found for FJ6. This is again partly due to the alteration in generation time (Table 4), but not entirely so. Whereas the ratio of the separation times for the wild type at 17 and 35°C is 5.3, for FJ3 it is only 2.5 (see Table 6).

Comparison of the rates of autolysis of native walls from FJ3 grown at 17 and 35°C (Fig. 6) shows that from the 17°C culture the walls lysed about five times more rapidly at pH 8.0 than did those from bacteria grown at 35°C. Likewise, extraction of cells grown at 17°C with 5 M LiCl yielded preparations that were also more active against SDS-treated walls from *B. subtilis* 168 *trpC* at pH 8.0 or against those from *M. luteus* at pH 5.6 than were extracts from cells grown at 35°C. The difference in activity between the extracts from cells grown at the two temperatures was about 5 to 10 times greater in the pH 8.0 test and 15- to 20-fold greater in the pH 5.6 test for the bacteria grown at 17°C rather than at the higher temperature. When inactivated walls prepared from the mutant grown at 17 and 35°C were incubated with 5 M LiCl extracts from strain 168 *trpC*, they were found to be equally susceptible when tested at pH 8.0. No evidence has so far been found to show that the enzymes formed at 17°C are themselves more susceptible to heat inactivation at 50°C than are the enzymes formed by strain 168 *trpC2*. Thus, it would seem likely that FJ3 is temperature sensitive in the production of its autolytic enzymes and in this property differs from FJ6.

No autolytic activity can usually be demonstrated if SDS-treated cell walls from susceptible microorganisms are incubated with the supernatant fluids from exponentially growing cultures of bacilli which are themselves exceedingly autolytic (19, 44). Nevertheless, if mutants deficient in the ability to turn over their wall are grown either in the presence of soluble autolysin from another organism or in mixed culture with the lytic organism itself, then the deficient mutants will undertake wall turnover (37, 44). This observation suggests that autolytic enzymes can be transferred from one cell to another during contact. Therefore, the effect of growing the wild type with the mutant strains FJ3 and FJ6 upon the length of the chains of unseparated mutant bacilli was examined. To do this, use was made of the different auxotrophic requirements of strain FJ6 and strain 168 *trpC2*. Three cultures, A, B and C, were set up in MSM medium supplemented by arginine

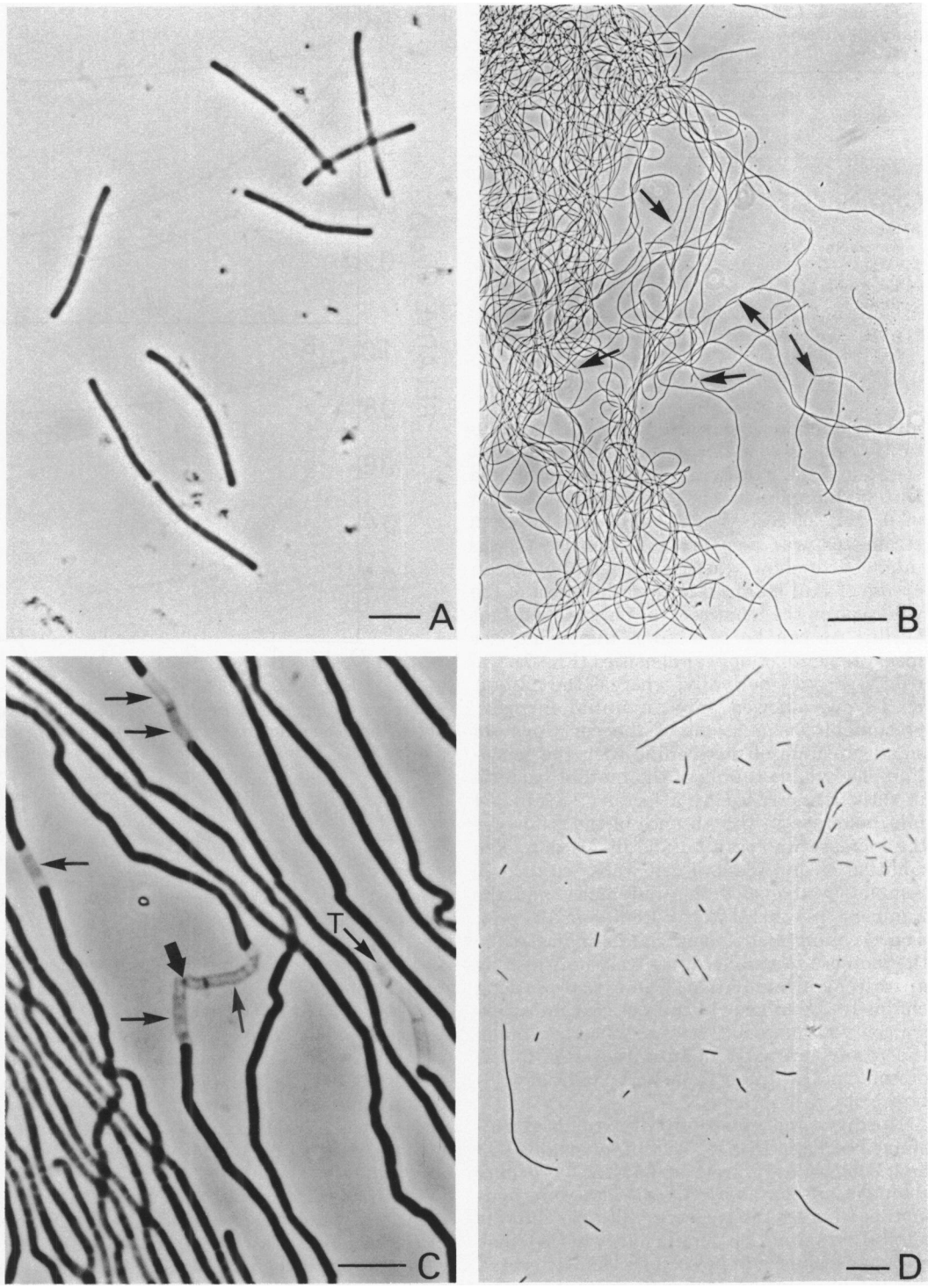

FIG. 5. *Strains FJ8 (A), FJ6 (B), and FJ3 (C) growing exponentially at 35°C in Pen B. The chains were often observed to clump together (B). Many chains contained one or more apparently empty, phase-light cells (arrows). Empty cells and wall fragments were also observed attached to a high proportion of the cells forming the termini of the chains (T). In (C), one chain possibly undergoing breakage is indicated (wide arrow). (D) Strain FJ3 grown at 17°C. Bars represent: (A and C) 5 μm, (B) 100 μm, (D) 20 μm.*

TABLE 6. *Calculated separation times for the autolytic enzyme-deficient mutants and their isogenic wild type*[a]

Medium	Growth temp (°C)	Separation time (min)		
		Strain FJ3[b]	Strain FJ6[b]	Strain FJ8[c]
Pen B	17	391	1,426	226
Pen B	35	158	169	50
SMM	35	245	241	38
SMM + L-glutamate	35	276	257	57
MSM	35	368	382	54
MSM + L-arginine	35	383	384	98

[a] The calculations were by the method of Sargent (45).
[b] Lyt⁻ mutant.
[c] Isogenic wild type.

and tryptophan. A was inoculated with the mutant strain, B was inoculated with the mutant strain and the wild type, and C was inoculated with both organisms, but a final concentration of 0.8 mg of SDS-treated walls per ml from strain 168 was also present. Culture C was intended to show whether the addition of an excess of wall material altered the effect of the wild type on the lengths of the chains of mutant bacilli. As can be seen from the results for measurements of optical densities (Fig. 7), the wild type grew normally, whereas the mutant on its own showed only an initial increase, presumably as a result of the carry-over of small amounts of methionine with the inoculum. The viable counts of the mutant bacteria in flask B increased by a factor of 7.9 in 174 min, whereas in the absence of the wild type the increase was only 2.1-fold in 203 min. The addition of an excess of cell walls (culture C) completely abolished this difference, and the numbers only increased by 1.8-fold in 190 min. The breaking of the long chains indicated by the increase in viable counts was confirmed by microscopic examination. There were no long chains in the mixed culture not containing extra cell wall material. It was difficult to make the results precisely quantitative, due to the presence of the numbers of short chains of wild-type cells.

Motility and susceptibility to bacteriophages ϕ29 and PBSI. The mutant strains FJ3 and FJ6 not only grew at 35°C as very long chains of unseparated cells but also were nonmotile, whereas the isogenic wild-type FJ8 was motile. Examination for flagella showed that although none could be seen on the bacteria of the autolytic enzyme-deficient mutants, numbers of flagella were present on those of strain FJ8. The mutant strain FJ3 grew at 17°C in Pen B medium, however, as very short chains, and produced autolytic enzymes. It was motile under these conditions and was susceptible to the bacteriophage PBSI. When phage PBSI grown on strain FJ3 at 17°C was used to infect BD112, prototrophs could be isolated, indicating that transduction had occurred. Examination of the susceptibility of strains FJ1, 2, 3, 6, and 8 to bacteriophage ϕ29 showed that they were all susceptible, as would be expected if the acceptor for this bacteriophage is the glucose of teichoic acid (63).

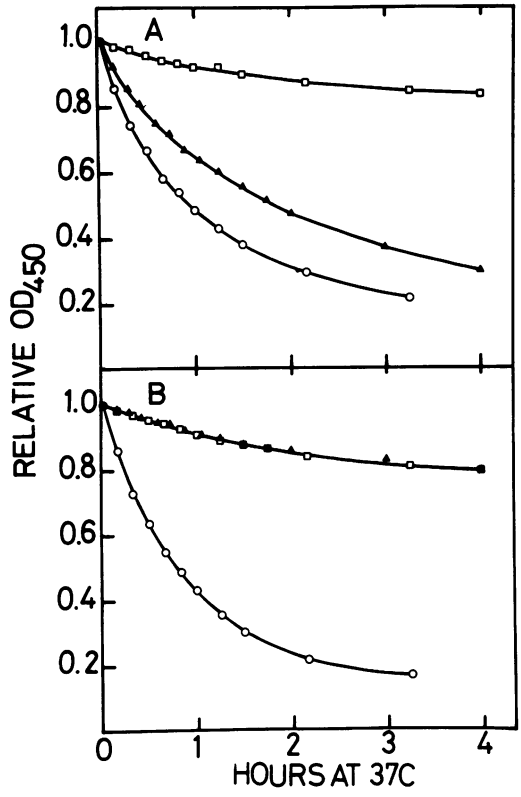

FIG. 6. *Comparative autolysis of native walls isolated from the mutant and wild-type strains grown at 17 and 35°C. Walls suspended in TL buffer containing 0.01 M $MgCl_2$ (final ionic strength, 0.1) were isolated from exponential-phase cells grown in Pen B at 17°C (A) and at 35°C (B), using the standard procedure. Symbols: ▲, FJ3; □, FJ6; and ○, FJ8. The rates for wall lysis for FJ3, FJ6, and FJ8 grown at 17°C were 4.1, 1.0, and 6.3 U and 0.8, 1.0, and 7.1 U when grown at 35°C, respectively. OD_{450}, Optical density at 450 nm.*

Phosphoglucomutase activity. Previously described autolytic enzyme-deficient mutants of *B. licheniformis* (18, 19) proved to be deficient in phosphoglucomutase. Although the wall chemistry of strains FJ3 and FJ6 was normal, glucose was present, and they were susceptible to bacteriophage ϕ29, it seemed desirable to measure the phosphoglucomutase activity in the cytoplasm of disrupted cells. Both FJ3

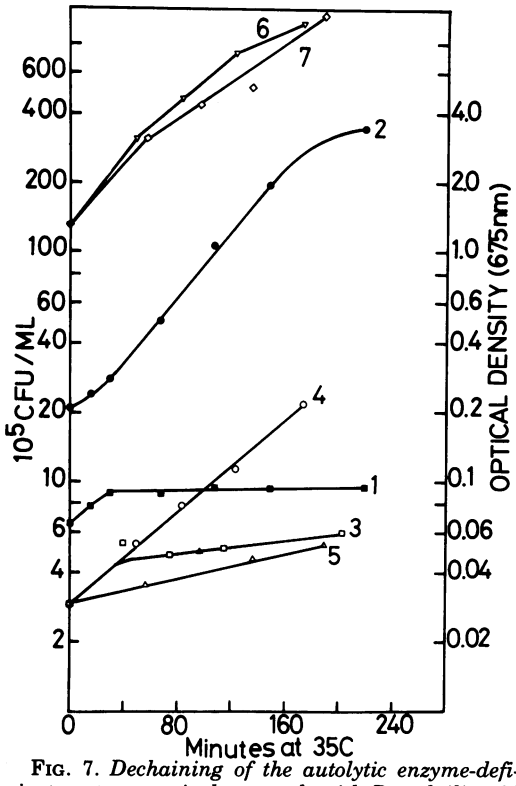

FIG. 7. *Dechaining of the autolytic enzyme-deficient mutant strain by growth with B. subtilis 168 trpC2. Strain FJ6 and the wild type were grown to a point somewhat past midexponential phase at 35°C in MSM supplemented with L-arginine and either 10 μg of L-methionine per ml (for FJ6) or 20 μg of L-tryptophan per ml (for B. subtilis 168). Three cultures were then set up, each containing MSM supplemented with arginine plus 20 μg of L-tryptophan per ml. Culture A was inoculated with strain FJ6, culture B was inoculated with both FJ6 and the wild-type strain, and culture C was as B, but with isolated SDS-treated walls (0.8 mg/ml) from the strain 168 added. Before inoculation the cell chains of the mutant strains were separated from each other by repeatedly drawing the culture through a pipette. The initial optical densities of the cultures containing mutant and wild-type strains were 0.065 and 0.14, respectively, and their chain lengths were 78.1 ± 48.4 cells and 8.3 ± 8.1 cells. The three cultures were incubated with shaking at 35°C. Growth was measured by the optical density of A and B; this was not meaningful with culture C, owing to the presence of the SDS-treated walls. Colony-forming units (CFU) of FJ6 were measured on solidified MSM supplemented with L-arginine, citrate (0.1%), and L-methionine (40 μg/ml). The wild type did not grow on this medium. The latter was enumerated by plating cultures B and C on medium, supplemented with L-tryptophan (20 μg/ml). Curves 1 and 2, optical density in culture A (1) and B (2); curves 3 to 7, plate counts for strain FJ6 in A (3), B (4), and C (5) and strain 168 in B (6) and C (7). At 203 min, the average chain length for strain FJ6 in flask A was 74.8 ± 40.8 cells.*

and FJ6 had high levels of the enzyme similar to that of the isogenic wild-type FJ8.

Protease and amylase formation. Ayusawa et al. (6) and Yoneda and Maruo (62) showed that the lesions in their partially autolytic enzyme-deficient mutants of *B. subtilis* were pleiotropic. They were hyperproducers of protease and amylase and were nonflagellated, despite the likely presence of an internal pool of flagellin. It was therefore important to look at these characteristics in the mutants described here. No evidence could be found that the autolytic enzyme-deficient mutants FJ3 and FJ6 were hyperproducers of protease or amylase. The activities of extracellular protease formed by sporulating cultures tested under the conditions of Dancer and Mandelstam (10) were closely similar to that of the isogenic wild type. The diameters of zones of hydrolysis produced by colonies grown on either skimmed milk or casein agar plates and on starch agar plates were also closely similar when the three strains were compared.

Transformability of strains FJ3, 6, and 8. The growth of strains FJ3 and 6 in very long chains of unseparated cells made quantitation by the usual methods of their competence to transformation very difficult. A rough test was therefore devised, in which the two mutants and the corresponding isogenic wild type were grown to approximately equal optical densities (A_{675} values of 1.250, 0.996, and 0.840, respectively, for FJ3, 6, and 8) in pretransformation medium (4). Equal volumes of these cultures were then transferred to the transformation medium and DNA isolated from FJ1 was applied to them. Transformants to prototrophy arising by transformation were counted. When related to a unit of optical density in the original cultures, the numbers were 3.18×10^3, 4.86×10^3, and 4.0×10^3, respectively, for FJ3, 6, and 8. Thus, within the limits of this test the two autolytic enzyme-deficient mutants appeared to be as readily transformed as the isogenic wild type. Examination of the transformants showed them to be autolytic enzyme deficient and to grow in Pen B medium as long chains of unseparated cells. Thus, the transformants could not have arisen from revertants of the mutant strains.

Reversion studies. Three spontaneous revertants of FJ3 (FJ16, 17, and 18) and one from FJ7 (FJ23) were obtained by selecting colonies of motile organisms among those of nonmotile ones. To do this, the mutant strains were plated on Pen B medium solidified with 0.5% agar. In this soft agar medium growth of motile organisms could clearly be distinguished by finger-like protrusions, as compared with the compact growth of the nonmotile organisms. The pheno-

type of all four organisms thus selected and characterized was Met⁻ and wild type as far as autolysin formation was concerned. They did not form long chains of cells but grew as singlets or doublets. Extracts made by LiCl from these isolates were all active in lysing SDS-treated walls from strain 168 at pH 8.0, presumably due to the presence of *N*-acetylmuramyl-L-alanine amidase, and in lysing *M. luteus* walls at pH 5.6, presumably due to the presence of β-*N*-acetylglucosaminidase, but the degree of activity seemed to differ from one revertant to another. FJ16 and 17 arising from FJ3 were particularly active (Fig. 8), but quantitative differences were not pursued further.

Induction of PBSX in the mutant strains. A mutant of *B. subtilis* 168, Ni15, is deficient in the production of PBSX, cannot turn over its walls normally (19, 44), and is partly deficient in autolysins. It was of interest to see whether PBSX could be induced in strains FJ3 and 6. Accordingly, mitomycin C (2.5 mg/ml) was added to cultures of Ni15, FJ3, FJ6, and FJ8 exponentially growing on Pen B, and they were incubated at 35°C for 16 h protected from light. On the next day the cultures were spotted onto nutrient medium grown with lawns of *B. subtilis* strain W23, which PBSX can lyse, whereas it is unable to lyse strain 168 (35). When the plates were incubated overnight at 30°C, zones of lysis were produced around the spots containing strains FJ3, FJ6, and FJ8, but not around that containing Ni15. No lysis was obtained when the mitomycin-treated cultures were placed on lawns of strain 168. Thus, it would appear that strains FJ3 and FJ6 are, unlike Ni15, not defective in PBSX.

Action of antibiotics on the autolytic enzyme-deficient mutants. It has been observed previously that autolytic enzyme-deficient bacteria neither lyse nor are killed readily by the addition to rapidly growing cultures of antibiotics that inhibit cell wall synthesis (43, 57, 58). Most of these previously examined bacteria had walls with altered composition that were not readily lysed by the wild-type autolysin. It was therefore of interest to examine the present autolytic enzyme-deficient mutants in this respect, since they have reduced autolysin but normally susceptible walls. Figure 9 shows the results of the addition of methicillin, cloxacillin, and ceporin to exponentially growing cultures of FJ7 and its isogenic wild-type FJ8. It will be seen that lysis, as measured by reduction of the optical density of the cultures, is less with the mutant strain than with the wild type. This is particularly true for the lower concentrations of methicillin and cloxacillin. With higher concentrations, which are great as compared with the minimal inhibitory concentration for these antibiotics, the differences between mutant and wild type became relatively smaller. At both of the concentrations of ceporin tried, the difference was rather slight.

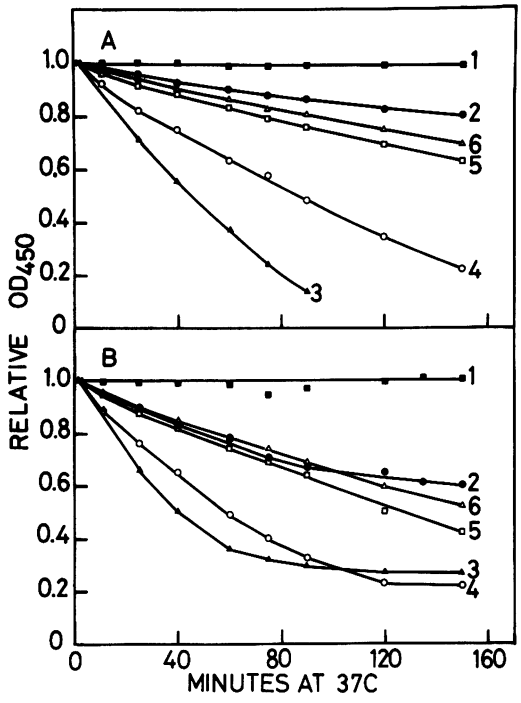

FIG. 8. *Lysis of B. subtilis 168 and M. luteus SDS-treated walls with crude LiCl extracts made from strains FJ3 and FJ8 and revertant strains FJ16, FJ17, FJ18, and FJ23. Cell pastes of viable cells were extracted with cold 5 M LiCl (approximately 400 mg of cell dry weight per ml). The extracts were diluted to contain 1.25 M LiCl and mixed with the SDS-treated wall suspensions in cold buffer. Wall lysis was initiated by transferring the suspensions to a 37°C water bath. (A) Lysis of B. subtilis 168 walls at pH 8 in TL buffer containing 0.01 M $MgCl_2$. The rates for wall lysis were 0.08 U (FJ3, curve 1), 1.04 U (FJ8, curve 2), 7.30 U (FJ16, curve 3), 4.44 U (FJ17, curve 4), 2.13 U (FJ18, curve 5), and 1.52 U (FJ23, curve 6). (B) Lysis of M. luteus cell walls at pH 5.6 in 0.05 M acetate buffer containing 0.01 M $MgCl_2$; designations are as in (A). Rates of wall lysis were >0.1 U (FJ3), 2.2 U (FJ8), 8.61 U (FJ16), 5.83 U (FJ17), 3.09 U (FJ18), and 2.70 U (FJ23). OD_{450}, Optical density at 450 nm.*

Spore formation and germination. In view of the possible involvement of autolytic enzymes in the germination of spores, the ability of the autolytic enzyme-deficient mutants to sporulate and germinate was examined. Strains FJ3 and FJ8 were first grown at 42°C in a casein hydrolysate medium and then transferred to a salts-glutamate medium (53). The

numbers of phase-dark bacteria and phase-light spores were counted at various times during incubation (42°C) in the sporulation medium. In one experiment after 6.25 h, 91% of FJ3 cells and 57% of FJ8 isogenic wild-type cells had spores within them. Spores from the two autolytic enzyme-deficient mutants grown on Schaeffer nutrient agar (47) also germinated at rates quite similar to those from the isogenic wild type (Fig. 10). In two experiments, spores from FJ3 germinated at a somewhat slower rate than those from strains FJ6 and FJ8. The difference was small, however, and of unknown significance. When the spore suspensions from the three strains were diluted onto nutrient agar immediately after heating for 15 min at 80°C, 98 to 100% formed bacterial colonies after 24 h of incubation of the plates at 35°C.

DISCUSSION

There is general agreement in the literature from the earliest observations with *S. faecalis* (31, 32) to the present work with *B. subtilis* that one action of bacterial autolytic enzymes is to help separate the bacteria from each other. If the enzymes are not normally active, the cells remain stuck together, as has been demonstrated for *Diplococcus pneumoniae*, *S. faecalis*, *B. licheniformis*, and *B. subtilis* (12, 18, 19, 38, 41, 56). The present mutants of *B. subtilis* are extreme examples of this phenomenon, forming chains of 200 or more cells on media in which they grow fast. In these mutants this is more likely to be a direct effect of the reduction in the activity of the enzymes in the presence of walls of normal chemistry and susceptibility. In other examples, the walls have known changed chemistry and are resistant themselves (18, 19, 26, 56) or have unknown composition and susceptibility (12, 13). This function for the autolytic enzymes must be of considerable consequence to the ecological survival of the organism in allowing dispersion in its habitat. Long strings, such as is the growth form of the present mutants, might soon exhaust their local nutrient supply and pathogenic species might suffer a loss of invasive powers.

The relationship of the autolytic enzymes to the rate of growth of bacteria in terms of mass, to their septation, and to their morphology has become rather confused, due largely to the observation of strains of *B. subtilis* investigated by Fan and his colleagues (12–15, 17). These

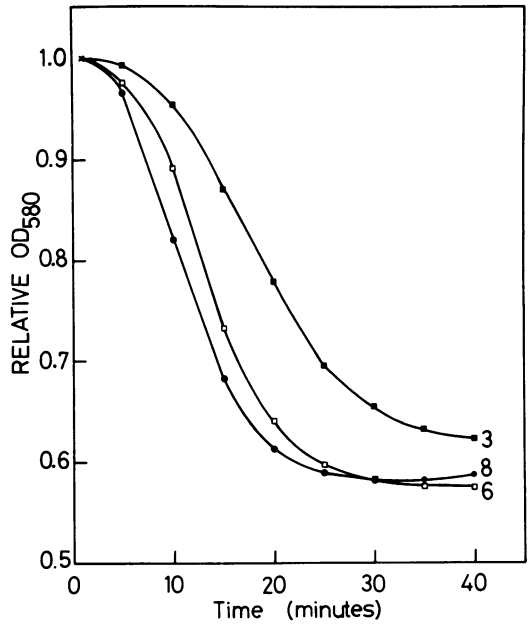

FIG. 10. *Germination of spores from strains FJ3 (■), FJ6 (□), and FJ8 (●) in Pen B at 37°C. OD_{580}, Optical density of 580 nm.*

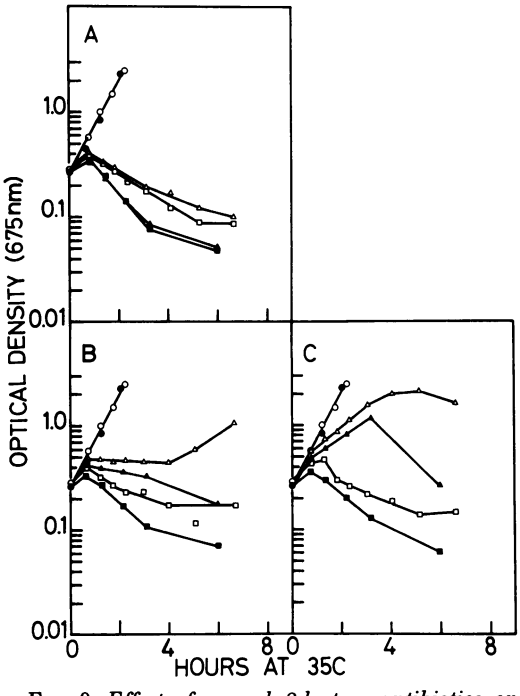

FIG. 9. *Effect of several β-lactam antibiotics on growth of strain FJ8 and an autolytic enzyme-deficient mutant, FJ7. At 0 min on the graphs, exponentially growing cultures of FJ8 (closed symbols) and FJ7 (open symbols) in SMM supplemented with 0.3% L-glutamate and L-methionine (20 μg/ml) at 35°C were treated with ceporin (A), methicillin (B), or cloxacillin (C). Symbols: ● and ○, no addition; ▲ and △, 0.5 μg of antibiotic per ml; ■ and □, 5 μg of antibiotic per ml.*

strains, called βA177 and βA203, were derived from another, βA175, which in turn was derived from a derivative, A0 (12) or βA0 (14), of *B. subtilis* 168. Each derivation involved drastic treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, followed by very intense selection pressure, first to obtain good growth at 51°C, the maximum growth temperature for the strain A0 or βA0, then for absence or reduced lysis at 51°C, and finally for better growth in the presence of autolysin. The parent strain itself grew as filaments or chains of unseparated cells at 48°C (12). No revertants of βA177 could be obtained and no attempt was made to transfer the genes concerned to other better-recognized strains. The strain βA177 grew as deformed, twisted rods at 51°C and was partially deficient in autolysin, and its rate of growth at 51°C was somewhat increased by the addition of either crude autolysin preparations or lysozyme. In view of the behavior of the original strain and of the history and selection procedures used, correlations and experiments cannot be used in any way to prove the causal relationship of autolysin production to growth or to the morphology of the bacilli. The present work makes it entirely unlikely that the degree of autolytic enzyme deficiency in strain βA177 would affect mass growth rate or the morphology of individual cells.

The autolytic enzyme-deficient mutants described in the present work reverted readily, and the revertants had lost all the phenotypic characteristics, having wild-type properties in these respects. The mutants studied in detail (FJ3, FJ6, and FJ7) were the result of transformation of a well-recognized strain with DNA from the original mutants isolated. They were compared with a constructed isogenic wild type, they were derived by minimum mutagenesis with ethyl methane sulfonate, and they were isolated without the application of known selection pressures, except for the addition of methicillin. Although these facts do not rigorously exclude involvement of multiple mutations, they make it much less probable. The mutant strains possessed not more than 5 to 10% of either the *N*-acetylmuramyl-L-alanine amidase or the β-*N*-acetyl-glucosaminidase, but grew at approximately the same rate as did the isogenic wild type in a variety of media. The morphology of the individual cells in the chains of unseparated bacilli appeared normal by light microscopy. Therefore, deprival of these two autolytic enzymes, to the extent found, affects neither the mass growth rate nor the individual cell morphology of *B. subtilis* 168.

It is of interest to note that FJ3 and FJ6 appeared to be competent as recipients of DNA in transformation experiments. Some work (1, 2, 5, 39, 48, 64) has suggested a role for the autolytic enzymes of pneumococci, streptococci, and bacilli in such competence. Lacks (30), however, had noted that one of his pneumococcal mutants was nonlytic but still competent. The present observations certainly cannot be construed as proof that they are not so involved. Nevertheless, in *B. subtilis* the autolytic activity can be reduced by at least 90% without apparent consequences. Perhaps further attention to the problem is necessary before their positive role can be accepted in this species. The importance of one or both autolysins as factors allowing the emergence of flagella is now supported by the present work and by that of Ayusawa and his colleagues (6, 62). It was possible to select autolysin-producing revertants by picking colonies of motile cells from strains FJ3 and FJ7. Other studies (J. E. Fein, unpublished data) confirm the correlation between autolysin deficiency and nonmotility in the phosphoglucomutase-deficient mutants of *B. licheniformis* (18, 19). Thus, not only do the autolysin-deficient mutants grow as long chains of cells, but they are also nonmotile, being a further negative factor preventing the dispersal of such bacteria in nature.

The breakdown of cortical peptidoglycan and the excretion of peptidoglycan fragments are well-recognized events occurring during the germination of spores. For this to happen "autolysin"-like enzymes must be present. Likewise, during the last stages of sporulation the wall of the vegetative cell has to be broken down to allow the escape of the formed spore. It was therefore possible that sporulation and germination might be affected in bacteria, such as the present mutants, that are grossly deficient in autolytic enzymes. Both strains formed heat-resistant spores that germinated in rich media at about the same rate as those from the isogenic wild type. Again, dogmatic conclusions cannot be drawn because of the small, residual autolytic activity in the mutants, but the results may favor the special switching on of autolytic enzymes during germination, rather than the initiation of the formation of the usual, vegetative cell enzymes.

Although the evidence from transformation and reversion studies would make the involvement of more than a single gene rather improbable, two enzymes were deficient in the mutants. The *N*-acetylmuramyl-L-alanine amidase is not particularly sensitive to proteolytic enzymes, although the β-*N*-acetylglucosaminidase is exquisitely so. Since both enzymes are reduced and no evidence for greater protease production by the mutants could be found, it

would seem unlikely that this is the explanation for deficiency in the autolysins. The mutant strains may be either regulation mutants or changed in the export of the enzymes. Further genetic and biochemical work is necessary to answer these questions.

The strains FJ3 and FJ6 were somewhat more sensitive to the autolysis that occurs when antibiotics inhibiting wall synthesis are added to exponentially growing cultures of these mutants than were autolytic enzyme-deficient pneumococci (57–59) or *B. licheniformis* strains (43). This may be because in the earlier work not only was the activity of the autolytic enzymes reduced but also either the walls of the bacteria had a changed composition or the strain hyperproduced lipoteichoic acid, so that the walls were resistant to the action of the lysins or the lysins were inhibited in situ. In the present mutants we are likely to see only the results of directly lowering the activity of the autolytic enzymes. It seems unlikely that the present mutant strains hyperproduce inhibitors of the autolysins, in view of the dechaining effect of growing the wild type with them. The cell-to-cell contact transfer of enzyme under these circumstances would be likely to involve only minute amounts of enzyme, but despite this the lengths of the chains of cells were rapidly decreased.

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