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Autolysins and Shape Change in *rodA* Mutants of *Bacillus subtilis*

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The biochemical phenotype of *rodA* mutants was not affected by the simultaneous presence in double mutants of the *lyt* gene which makes them 90 to 95% deficient in autolysin action. The only morphological effect of this deficiency on the expression of the *rod* gene was that both the rod and the coccal forms of the mutant failed to separate and grew as long chains of cells. Inhibition of protein synthesis stopped the increase in peptidoglycan that occurred when the growth temperature for the mutants was raised to 45°C. These observations support the idea that a derepression of peptidoglycan synthesis occurs at this temperature. The increased amount of cellular peptidoglycan at the higher growth temperature is not likely to be the result of the concomitant switching off of autolytic enzyme action.

When grown at a temperature of 42 to 45°C, the *rodA* mutants of *Bacillus subtilis* (3, 15) change from normal rods to groups of misshapen spheres. This change is accompanied by a fall in the proportion of glycerol teichoic acid per unit weight of walls and an almost complete elimination of the *N*-acetylgalactosamine polymer (4). There is also a large increase in the cellular proportion of peptidoglycan (23), which is nearly sufficient to account for the decrease in the wall glycerol teichoic acid by dilution with peptidoglycan. To achieve this result, peptidoglycan must accumulate while teichoic acid either does not do so at the same rate or, if it does, is specifically eliminated. One possible explanation (27) would be a reduced activity of the autolysins together, presumably, with some method for specific elimination of the teichoic acid. The wall of the exponentially growing wild type is known to turn over (17, 18, 19), and if the removal side of this balanced process is stopped by the absence of the action of appropriate autolytic enzymes, an increase in the proportion of wall polymers in the cell would be expected. It could be questioned whether an increase of three- to fivefold in the two or three generations of growth could be thus explained. It is known, nevertheless, that, after the change in growth, there is a gross reduction in the autolytic activity demonstrable in the cells of *rodA* mutant strains (4, 5). This reduction appears to be due to a change in the walls, which become a poor substrate for the enzymes, as well as possibly to a failure to produce active enzymes (5).

In the present work, wall synthesis was studied in double mutants constructed from the *rodA* strains and two autolytic-deficient ones. The effect of inhibiting protein synthesis upon peptidoglycan formation during the switch in growth temperature was also explored. If the failure in autolysin formation at 45°C were the sole explanation for the increase in peptidoglycan synthesis, continued protein synthesis might be expected to be irrelevant.

MATERIALS AND METHODS

Microorganisms. The strains used are summarized in Table 1.

Media. The media used were as follows. (i) A casein hydrolysate-yeast (CHY) (14), which was supplemented with D-alanine (40 μg/ml) when used for the growth of the mutant strain *rodA ald*. (ii) A salts-glucose medium (MMS) (24), which was supplemented when necessary with one or more of the amino acids L-leucine, L-isoleucine, and L-methionine (all at 40 μg/ml) to meet the auxotrophic requirements of the various strains. (iii) A cell wall synthesis medium, which was a solution containing as final concentrations: 0.02% DL-alanine, DL-aspartate, and DL-glutamate; 2 mM $MgSO_4$; 1% glucose; and 0.1 M KH_2PO_4-NaOH (pH 7.0). Chloramphenicol was included in certain experiments at a concentration of 10 μg/ml to inhibit protein synthesis (see Results).

Conditions and measurements of growth. Cultures of up to 100 ml were grown in side-arm flasks incubated at 35°C and shaken to obtain aeration. Larger volumes needed for the preparation of cell walls or for the study of autolysins were grown as 11 batches in 5-liter flasks, again at 35°C with shaking. Growth was measured by the extinction values of the cultures at 675 nm.

Transformation. Transformation was done as previously reported (7), including the use of the Procion red cell wall agar for determining the autolytic ability of colonies.

TABLE 1. *Description of B. subtilis strains*

Strain	Genotype	Phenotype	Source or reference
172ts 200B	*leu8 metB5 rodA*	Leu⁻ Met⁻ Rod⁻	Transformation 172/200B[a]
FJ3	*metC lyt*	Lyt⁻ Met⁻	(7)
230	*metB5 lyt rodA*	Met⁻ Lyt⁻ Rod⁻	Congression (P. J. Piggot, C. Taylor, P. F. Thurman, and H. J. Rogers, unpublished data)
RB973	*rodA gtaC51*	Rod⁻ Pgm⁻	Transformation 172/BC7
BC7	*gtaC51 hisA1 cysB3*	Pgm⁻ His⁻ Cys⁻	Transformation BY51/BD92, B. E. Reilly
rodA ald	*rodA ald*	Rod⁻ D-Ala⁻	(23)
172 (BD54)	*leu8 ile-1 metB5*	Leu⁻ Isoleu⁻ Met⁻	D. Karamata

[a] 200B is the mutant described by Boylan and Mendelson (3).

Preparation of SDS-treated cell walls. Cultures in the mid-exponential phase of growth were cooled to 5°C and centrifuged at 12,000 × *g* for 5 min. The bacteria were washed twice with ice-cold water and then lyophilized. The dried cells were suspended, when required, at 50 μg/ml in water at 4°C and passed two to three times through a well-cooled Aminco French pressure cell at 20,000 lb/in². Cell breakage was >95%. The suspension of broken cells was centrifuged at 27,000 × *g* for 10 min at 5°C to deposit the walls, which were mechanically separated from the small underlying deposit of unbroken cells. They were then suspended in 4% (wt/vol) sodium dodecyl sulfate (SDS), and the suspension was raised to 100°C, cooled to room temperature, and centrifuged at 27,000 × *g* for 10 min at 20°C. The walls were then subjected to the following washing schedule: (i) 1× 4% SDS, (ii) 2× 1 M NaCl, and (iii) 6× water. Finally, they were lyophilized.

Analysis of wall preparations. The lyophilized preparations were further dried to constant weight by heating at 105°C and placing under vacuum over P_2O_5. Known replicate quantities of about 2 mg were hydrolyzed for 2, 4, and 16 h in 2 N, 4 N, and 6 N HCl, respectively, at temperatures of 100°C for the first two concentrations of acid and 105°C for the last. All hydrolyses were conducted in sealed tubes. The acid was then removed under vacuum in a rotary evaporator, and the hydrolysates were suspended in a known volume of water. Those made in 4 N and 6 N acid were analyzed for amino sugars and amino acids, respectively, using the Beckman 120-C automatic amino acid analyzer. The 2 N acid hydrolysates were adjusted to pH 9.0 and a concentration of 0.05 M NH_4HCO_3. Alkaline phosphatase was added, and the samples were incubated for 2 h at 35°C to hydrolyze phosphate esters. They were then boiled for 5 min to precipitate the enzyme, which was removed by centrifugation. The supernatant solutions were analyzed for glycerol (26) and glucose (2). Dried wall samples were suspended (1 mg/ml) and examined for total phosphorus (1).

Estimation of cellular content of peptidoglycan. Known quantities of washed bacteria were dried to constant weight at 105°C, and hydrolysates were prepared as above in 4 N and 6 N HCl. The former were analyzed for total hexosamines (16) and the latter for 2,6-diaminopimelic acid by the Beckman 120-C automatic amino acid analyzer.

Autolysis of cells and cell walls. For autolysis of cells and cell walls, the treatments of the walls with SDS and NaCl were omitted. Lyophilized cells or cell walls were suspended to 0.5 to 1.0 mg/ml in ice-cold 0.05 M $(NH_4)_2CO_3$ buffer (pH 9.5) and in 0.05 M cacodylate-hydrochloride buffer (pH 5.5) and placed in 1-cm cuvettes. The *N*-acetylmuramyl-amidase was estimated by incubation at the high pH and the endo-β-*N*-acetylglucosaminidase was estimated at the lower pH. The extinctions at 450 nm of these suspensions were read immediately and again after 2 min of incubation at 37°C. Incubation was continued, and the extinctions were read at intervals over a period of 30 to 60 min. Autolytic activity was measured from the initial rate of reduction in extinction. One unit of activity is that amount of enzyme which causes a fall of 0.001 per min in a suspension with an initial extinction of 1.000.

Lytic activity of cell extracts. Bacteria from mid-exponential phase cultures in CHY medium were obtained by centrifugation and washed twice with ice-cold water before lyophilization. They were then suspended (100 μg/ml) in 5.0 M LiCl at 0°C for 45 min to extract autolytic enzymes, and the suspensions were centrifuged at 27,000 × *g* for 10 min at 5°C. The supernatant fluids were diluted 1:4 with water. Cell walls were prepared as substrates by SDS treatment as already described. Their lysis was measured as follows: 3.87 ml of a suspension of SDS-treated cell walls (0.25 to 0.5 mg/ml) in ice-cold 25 mM tetraborate buffer was adjusted to pH 9.5 with 100 mM NaOH and made to a final concentration of 20 mM $MgCl_2$. This suspension was mixed with 0.13 ml of the diluted extract and transferred to a 1-cm cuvette. The fall in absorbance at 450 nm (A_{450}) was measured during incubation at 37°C as in the measurement of the autolysis of cells and walls. Units were derived from the expression $\{[A_{450}\ (0\ \text{min}) - A_{450}\ (60\ \text{min})]/A_{450}\ (0\ \text{min})\} \times 100$.

Rates of peptidoglycan synthesis. Peptidoglycan synthesis was measured by the incorporation of *N*-acetyl-[1-^{14}C]glucosamine into the appropriate fraction from the cells (23). In the absence of protein synthesis it was measured by first growing the bacteria with unlabeled *N*-acetylglucosamine (23). The cells were then filtered from the cultures, washed, and suspended in medium C with chloramphenicol and with *N*-acetyl-[1-^{14}C]glucosamine present. Rates of incorporation over the first 30 min were measured. The small amount of apparent protein synthesis occurring in the presence of 10 μg of chloramphenicol per ml was measured by the incorporation of [4,5^{3}H]leucine present both during the preincubation and in the final

culture. The protein fraction from the cells was obtained as before (23).

Measurement of teichoic acid biosynthesis. The mutant strain *rodA ald* was grown in medium A supplemented with D-alanine, 0.05% glycerol, and 0.5% glucose at either 30 or 45°C. The inoculum was such that the organism grew exponentially for 16 h. At this time the culture was reinoculated into a fresh quantity of the same medium but also containing [2-^{3}H]-(n)glycerol at a final specific activity of 0.368 μCi/μmol. This culture was incubated for eight generations of growth before being subcultured into two separate batches of the same medium, one of which was at the 45°C and the other at the 30°C growth temperature according to the experiment. Duplicate samples (2 ml) were removed at frequent intervals, and the incorporation of glycerol into the teichoic acid fraction of the cells was measured by one of the two following methods.

Method 1. The samples were mixed with equal volumes of cold 10% (wt/vol) trichloroacetic acid to extract pool substances. The cells were filtered, washed, and suspended in 5% (wt/vol) trichloroacetic acid. The mixtures were heated to 90°C for 20 min and filtered through a glass-fiber disk. The disks and the deposits were washed with 1 ml of cold 5% (wt/vol) trichloroacetic acid. The combined filtrates and washings were extracted three times with equal volumes of diethylether, the aqueous phase was dried in vacuo, and the residues were dissolved in water. Samples were counted in a 1:4 mixture of Biosolve and toluene for ^{3}H. The incorporation of glycerol into teichoic acid was taken as the difference between the ^{3}H counts in the hot and cold trichloroacetic acid extracts.

Method 2. The samples from the culture were centrifuged and washed with 2 ml of cold unlabeled medium, suspended in 1 ml of water, and heated at 100°C for 5 min to extract the water-soluble pool. They were then centrifuged again, and the cells were washed with 1.0 ml of water. The cells were then lyophilized, extracted for 5 min with 1.5 ml of methanol at 65°C, and cooled to room temperature; 3 ml of chloroform was added; and the samples were shaken at frequent intervals during 30 min at room temperature. The residue was recovered by centrifugation and reextracted by the same method. This procedure was repeated twice more. The residues were dried in vacuo and suspended in 1 ml of CHY medium, and lysozyme at a final concentration of 100 μg/ml was added. After 2.5 h at 35°C, the lysozyme-solubilized fractions were recovered by centrifugation, dried in vacuo, suspended in known volumes in water, and counted for radioactivity. This fraction contained radioactivity from both teichoic acid and peptidoglycan, if the latter had been labeled by including D-[^{14}C]alanine in the growth medium.

RESULTS

The two double mutants, 230 and RB973, were different in physiology in that RB973 was deficient in phosphoglucomutase and 230 was deficient in some unknown regulatory gene controlling the formation of active autolysins (7). Nevertheless, both were grossly deficient in autolysin and at 30°C grew as long chains of unseparated nonmotile rods (Fig. 1A). When the growth temperature was changed to 45°C, the rods first changed to oblate spheres still remaining in chains (Fig. 1B). As incubation proceeded, these chains appeared to twist, until a disorderly mass of spherical-shaped cells was left (Fig. 1C). Examination of isolated native walls of the double mutants confirmed that very little autolytic activity was present in them when the bacteria were harvested from exponential cultures growing at either 30 or 45°C (Table 2). The walls from strain RB973 lysed at the same rate whether derived from bacteria grown at 30 or 45°C. The lytic rate was about 5 to 10% of that of the parent strain 172. The lytic activity of the walls in both strains RB973 and 230 after growth at 30°C was similar to that in strain 172ts 200B walls and *rodA ald* cells after growth at 45°C. Grown at 30°C, the activities of these latter strains are 10 times greater than after growth at 45°C (5). Attempts to measure lysis at pH 5.5, at which the second autolytic enzyme, β-*N*-acetylglucosaminidase, is active, showed that even with strain 172ts 200B, the values were so low at 30°C that accurate estimates were not possible, but it appeared probable that there was a reduction after growth at 45°C. In the two *lyt* double mutants, activities were altogether too low for statements to be made for either growth temperature.

The autolytic properties of the strain 230, the parent 172, and 172ts 200B were examined using the activity at pH 9.5 of 5 M LiCl extracts prepared from lyophilized cells upon SDS-treated cell walls. Again there was no evidence (Table 3) for a significant reduction in the amount of enzyme activity extracted from strain 230 when grown at 45°C instead of 30°C. The walls of this strain, grown at the higher temperature, are less susceptible to the small activity of residual enzyme in the mutant strain and to the large activity in the extract from the parent. This is similar to the results obtained for walls from RuB 1000 and RuB 1012, two other presumably *rodA* mutant strains (5). The activity of extracts and the susceptibility of walls when used as substrates were the same when obtained from the wild type grown either at 30 or 45°C. The wild-type cells, however, autolyzed less rapidly after growth at 45°C (Table 2). Walls from all the strains were isolated using SDS treatment, hydrolyzed, and chemically analyzed. Comparison of the results obtained from cells grown at 30°C with those from cells grown at 45°C showed (Tables 4 and 5) that the change in the walls from the double mutants was very similar both to that for the strain *rodA ald* and for strain 172ts 200B (23). The total phosphate of the walls of all three strains was reduced by

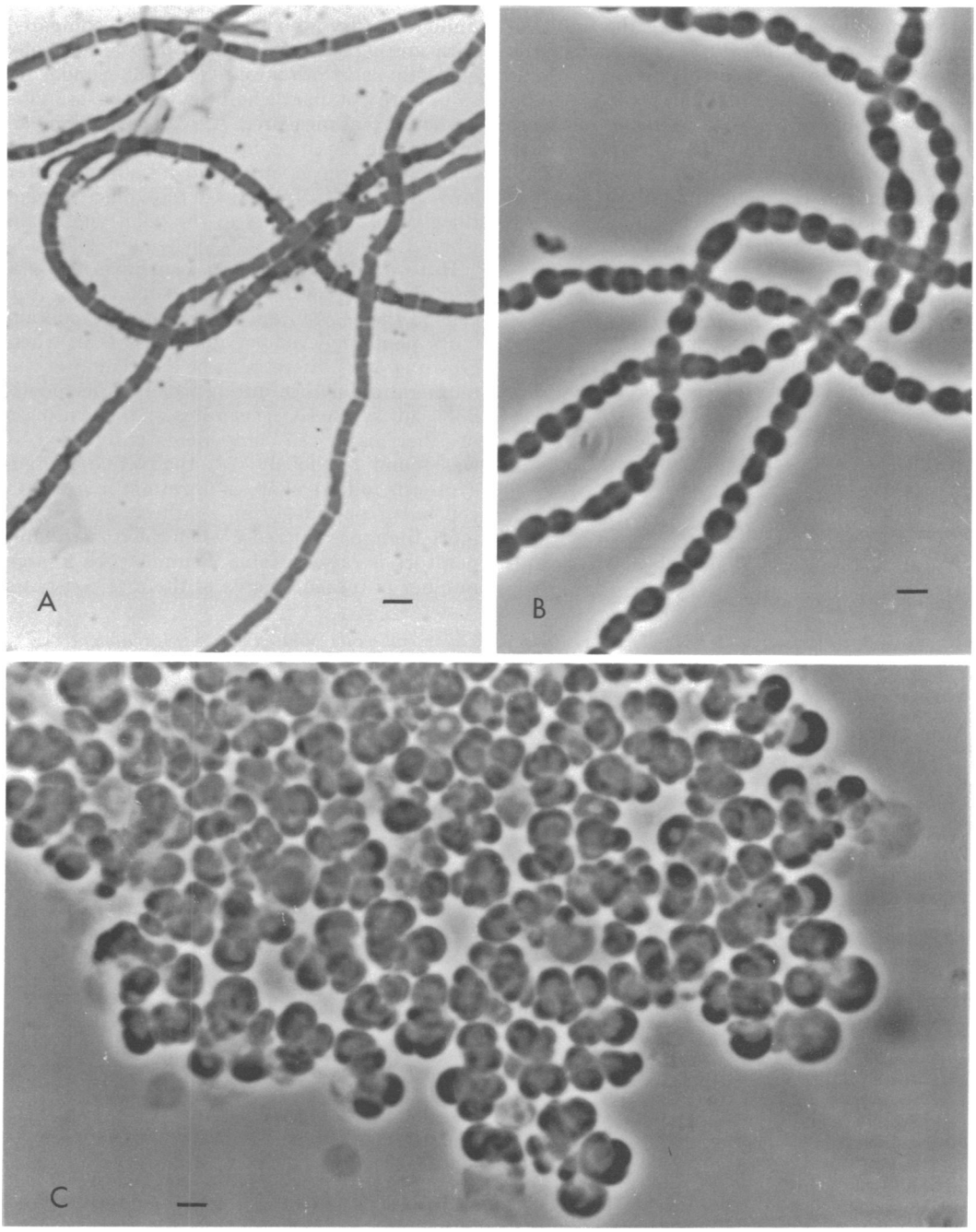

FIG. 1. *Morphological change in B. subtilis lyt rodA (strain 230) after growth temperature shift from 30 to 45°C. (A) Time 0; (B) after 80 min; (C) after 265 min. Bars represent 2 μm.*

80 to 90%. In strain 230, this was shown to be accompanied by similar decreases in the glycerol and glucose contents. The proportion of peptidoglycan components in the wall, on the other hand, was increased by about twofold. There was a close similarity in the increases of muramic acid and 2,6-diaminopimelic acid in the walls of all three strains. The somewhat smaller increase in muramic and diaminopimelic acid in strain RB973 after growth at 45°C may be accounted for by the absence of the galactosamine-glucose-phosphorus polymer at 30°C and the substitu-

tion of polyglycerolphosphate as the only teichoic acid at this temperature. This is due to the phosphoglucomutase-negative phenotype of this strain (Table 5). It was previously reported (23) that the amount of peptidoglycan per unit mass of cells increased in 172 *rodA* when the growth temperature for the organism was raised. Again, the two mutants deficient in autolytic enzymes behave almost identically, increasing 2.8 and 3.5 times in content of 2,6-diaminopimelic acid per milligram (dry weight) of bacteria, compared with an increase of 3.5 times for the normally autolytic *rodA ald* strain (Table 6). Similar results were obtained when the total hexosamine in the cell was measured. A small correction had to be made for the content of galactosamine in the cells grown at 30°C and its absence after growth at 45°C. No increase took place in peptidoglycan constituents in the wild-type strain when it was grown at 45°C instead of 30°C.

TABLE 2. *Autolytic activity of whole cells and native walls of B. subtilis mutants*[a]

Strain	Units of activity at growth temp:	
	30°C	45°C
rodA ald (cells)	31.5	2.8
172 (cells)	77.7	33.2
172ts 200B (walls)	41.3	3.97
RB973 (walls)	3.5	3.90
230 (walls)	4.6	2.26

[a] Walls and cells were incubated at 35°C and pH 9.5.

Rate of peptidoglycan synthesis after a switch in growth temperature. Since the cells of the *rod* mutants grown at 45°C contain more peptidoglycan per unit mass than when grown at 30°C, there must be a period after the temperature change during which either peptidoglycan synthesis accelerates or the synthesis of other major cell components is retarded. It was found previously (23) that while protein synthesis follows changes in rate of increase in cell mass, peptidoglycan synthesis accelerates more than mass increase when the growth temperature is raised. Table 7 summarizes a large number of measurements of the rate constants

TABLE 3. *Activity of 5 M LiCl extracts from cells upon cell walls isolated with SDS treatment*[a]

Source of extract, strain	Source of substrate, strain					
	230 (30°C)	230 (45°C)	172 (30°C)	172 (45°C)	172ts 200B (30°C)	172ts 200B (45°C)
230 (30°C)	4.8	1.5	6.3	1.9	ND	ND
230 (45°C)	5.2	1.6	0	3.3	ND	ND
172 (30°C)	89.2	33.6	86.0	84.0	93.9	36.3
172 (45°C)	95.8	45.9	93.9	86.3	96.5	47.0
172ts 200B (30°C)	70.0	31.4	60.0	63.9	69.6	26.0
172ts 200B (45°C)	16.5	4.5	9.2	24.6	25.8	6.3

[a] Figures in parentheses show the temperature at which the bacteria had been grown to produce either substrate or extract. ND, Not measured.

TABLE 4. *Chemical analysis of cell walls from B. subtilis strains*

Constituent	Analysis (nmol/mg of wall) of strain at growth temp:							
	172		*rodA ald*		230		RB973	
	30°C	45°C	30°C	45°C	30°C	45°C	30°C	45°C
Muramic acid	313	320	272	592	359	677	313	543
Glucosamine	ND[a]	ND	359	751	390	803	ND	ND
Galactosamine	ND	ND	118	0	172	9	ND	ND
Glutamic acid	401	421	344	851	437	878	483	711
2,6-Diaminopimelic acid	587	507	408	835	477	992	600	803
Alanine	702	774	618	1,346	890	1,536	861	1,182
Glycine	ND	ND	16.6	74	36.5	48.5	ND	ND
Total phosphate	1,637	1,482	1,152	187	1,316	205	2,000	252
Glucose	ND	ND	905	54.6	891	54.6	0	0
Glycerol	ND	ND	1,018	167	1,284	82.6	ND	ND
Ester alanine (chemical)	ND	ND	101	36	ND	ND	ND	ND
Ester alanine (radioactive)[b]	ND	ND	78.4	29.8	ND	ND	ND	ND

[a] ND, Not done.

[b] Estimated after incorporation of L-[^{14}C]alanine (see reference 23).

for the increases in mass and in peptidoglycan per milliliter of culture. Both strains 172ts 200B and RB973 had rate constants for peptidoglycan synthesis, after the growth temperature was changed from 30 to 45°C, of about twice the values of those for peptidoglycan increase in cultures growing steadily at 45°C. This greater rate lasted for about two generations before it subsided to the value for the culture maintained at 45°C (Fig. 2). The wild type showed no prolonged acceleration in peptidoglycan synthesis, although cultures frequently showed a short burst of more rapid synthesis, lasting for less than one generation. The autolytic-deficient *rodA* strains showed the same acceleration lasting for the same time, as the autolytically competent strain.

Synthesis of teichoic acid by *rodA* mutants. Previously (23) indications were obtained that the rate of teichoic acid synthesis might be relatively unaffected by the changing of growth temperature of *rodA* mutants from 30 to 45°C. At this time only the incorporation of [1-^{14}C]-alanine into the ester-alanine of the teichoic acid was measured. Calculations, however, from the figures given in Tables 4 and 6 show that, on a cell mass basis, the amount of peptidoglycan increases three- to fourfold but that the total amount of glycerol teichoic acid in the cell, measured as polyglycerolphosphate, falls by two- to threefold. To investigate this further, the incorporation of [2-^{3}H]glycerol into the teichoic acid fractions of the strain *rodA ald* was studied after a switch in growth temperature from 30 to 45°C. The cultures used for this experiment were allowed to grow through more than 12 generations in the medium before any switches in growth temperature were made or samples were taken. This was done both to ensure full induction of any necessary uptake systems for glycerol and to obtain uniform labeling of all the cell components. Incorporation into the teichoic acid fraction was followed by two methods (see Materials and Methods section). Results obtained using the more complicated but less ambiguous

TABLE 5. *Ratios of constituents of cell walls from B. subtilis strains after growth at 30 and 45°C*

Constituent	Ratio of constituent after growth at 45°C to that at 30°C in strain:			
	172	*rodA ald*	230	RB973
Muramic acid	1.02	2.18	2.20	1.75
2,6-Diaminopimelic acid	0.88	2.06	2.08	1.35
Total phosphate	0.91	0.16	0.16	0.13

TABLE 6. *Content of 2,6-diaminopimelic acid per unit cell mass of B. subtilis strains*

Strain	Content (nmol/mg [dry weight] of cells) at growth temp:		Ratio 45°C/30°C
	30°C	45°C	
rodA ald	73.3	253.4	3.5
230	79.5	222	2.8
RB973	153	542	3.5
172	71.0	70.0	0.99

TABLE 7. *Rates of peptidoglycan synthesis by B. subtilis mutant strains*[a]

Strain	Medium	Rate of synthesis[b]	Growth temp		
			30°C	30 and 45°C	45°C
172	MMS	G	0.49	0.98	1.18
		MP[c]	0.59	1.48	1.45
	CHY	G	1.21	2.5	2.5
		MP	1.41	2.3[d]	2.3
172ts 200B	MMS	G	0.46	0.95	0.94
		MP	0.51	1.89	0.84
RB973	MMS	G	0.35	0.78	0.92
		MP	0.36	1.54	0.84
	CHY	G	0.87	1.8	1.45
		MP	1.04	4.2	1.86

[a] The rates of peptidoglycan synthesis by the wild-type, *rodA* mutant (strain 172ts 200B) and a *rodA* autolytic enzyme-deficient mutant (strain RB973) during growth at 30°C, at 45°C, and after the transition from 30°C to 45°C. Peptidoglycan was measured from the *N*-acetyl-[^{14}C]glucosamine incorporated into isolated fractions as described in the text. The figures given are for rate constants (i.e., 0.693 divided by the doubling time in hours).

[b] The rate constant in h^{-1} for the increase in bacterial dry weight. MP, The rate constant in h^{-1} for the increase in the radioactivity of the isolated peptidoglycan fraction.

[c] Duplication in this and a number of other experiments with strain 172 growing in the mineral salts-glucose medium was poor.

[d] For a short initial period of less than one generation, the value was higher.

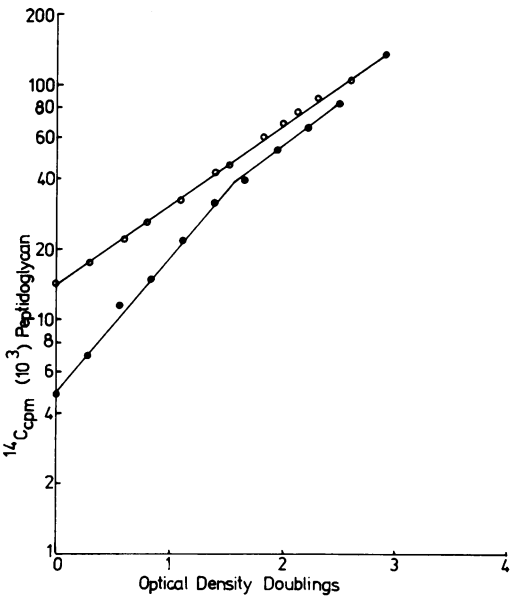

FIG. 2. *Effect on the synthesis of peptidoglycan by strain RB973 of switching the growth temperature from 30 to 45°C at time zero. Radioactivity of the peptidoglycan fraction:* (○) *after constant growth at 45°C;* (●) *after switching from 30 to 45°C.*

method of analysis (Method 2) were disappointing because of the great difficulty of obtaining reproducibility of the results using the necessarily very small masses of cells obtainable, particularly in the early stages of incubation. Low cell densities at the beginning of the experiments were necessary to allow exponential growth throughout. The results (Fig. 3) by either method of cell fractionation clearly confirm that there is a depression of about 50% in the total amount of teichoic acid in the cells as a result of altering the growth temperature from 30 to 45°C. This fall takes place very rapidly within the first generation of growth after the temperature change.

Relation of protein synthesis to increased peptidoglycan. It is well known that peptidoglycan synthesis by bacteria can proceed in the absence of protein synthesis, but that the formation of fresh autolytic enzyme cannot. Inhibition of protein synthesis, partly for this reason, prevents autolysis of *B. subtilis*. If the increased amounts of peptidoglycan found in *rodA* mutants after growth at 45°C were due to the disappearance of an autolytic system, the inhibition of protein synthesis and, hence, autolysin synthesis should not stop it.

In preliminary experiments, an unexpected difficulty was met because lysis occurred when chloramphenicol at 50 μg/ml was added to cultures of any of the strains of *B. subtilis* and the growth temperature was changed from 30 to 45°C. This did not happen when the temperature was maintained at either 30 or 45°C. Such a concentration of the antibiotic has commonly been used to inhibit bacterial protein synthesis without interfering with peptidoglycan formation. Surprisingly, the autolytic-deficient strain RB973 also lysed (Fig. 4). When the concentration of the antibiotic was lowered to 10 μg/ml or less, lysis did not occur at least for a considerable time (up to 2 to 3 hours). Incorporation of [4,5-^{3}H]leucine into strain 172ts 200B was studied with subsequent extraction of the protein fraction from the cells. Whereas the addition of 50 μg of chloramphenicol per ml to the cultures reduced incorporation of the amino acid to about 10% of the value of the control, 10 μg/ml also reduced it to about 10 to 15%. Chloramphenicol at 10 μg/ml also inhibited the incorporation of [2-^{3}H]methionine into the protein fraction by more than 90%. This lower concentration of the antibiotic could, therefore, be used to study the initial rates of synthesis of peptidoglycan by strain 172ts 200B after changing the incubation temperature from 30 to 45°C in the virtual absence of protein synthesis and without cell lysis. The strain was grown in medium B in the presence of 0.3 mM unlabeled *N*-acetylglucosamine, filtered through membrane filters (Millipore Corp.), and suspended in cell wall medium, together with *N*-acetyl-[1-^{14}C]glucosamine and [4,5-^{3}H]leucine. To compare the results for peptidoglycan synthesis obtained with and without the addition of 10 μg of chloramphenicol per ml, the results were plotted as the increase in the ratios of the radioactivities in the peptidoglycan to those in the protein fractions. The results obtained (Fig. 5) after the growth temperature was changed from 30 to 45°C showed that the increased synthesis of peptidoglycan did not occur when protein synthesis was inhibited. At steady growth temperatures of either 30 or 45°C, the addition of chloramphenicol to the culture had little effect on peptidoglycan synthesis, although the rate appeared somewhat less at 45°C in its presence.

The increased synthesis of peptidoglycan by the *rodA* mutants was blocked by inhibiting protein synthesis, but this did not entirely prevent the characteristic morphological change at 45°C. Only some bacteria changed to spheres, and even these were less regular in shape than usual. Nevertheless, a considerable increase in diameter of all the individual cells occurred. These changes took place in the presence of both 10 and 50 μg of chloramphenicol per ml as well as in the presence of other inhibitors of protein synthesis such as erythromycin, puro-

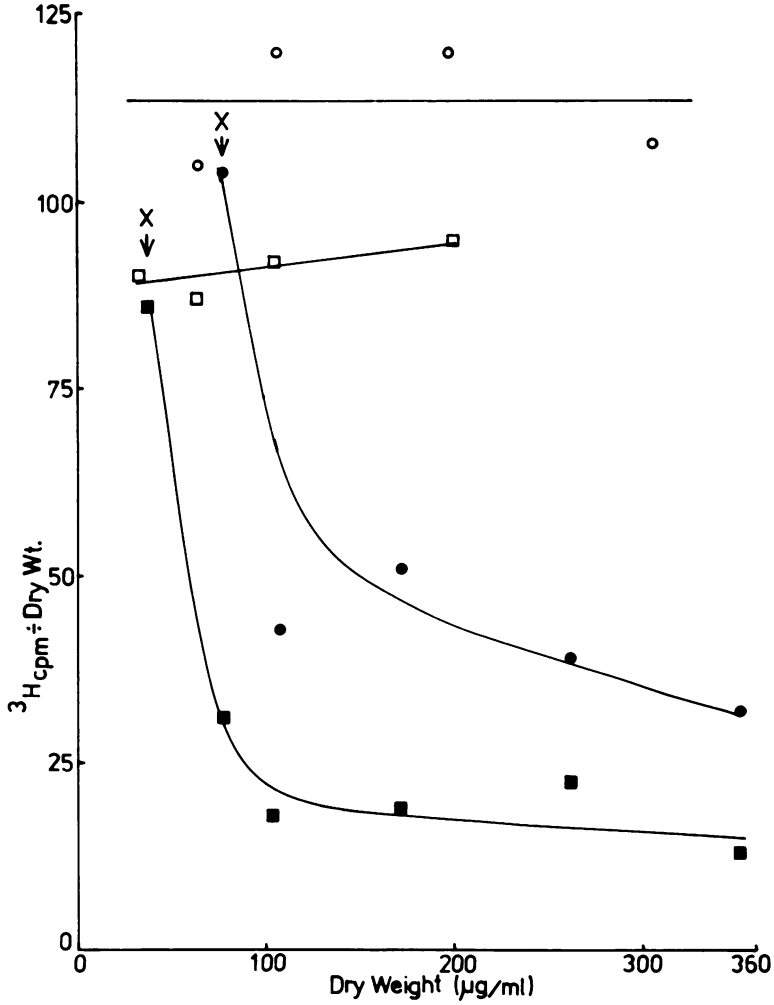

FIG. 3. *Effect of changing the growth temperature for strain rodA ald from 30 to 45°C upon the incorporation of [2-^{3}H]glycerol into the wall teichoic acid fraction.* (○, □) *Growth at 30°C;* (●, ■) *growth temperature changed from 30°C to 45°C at time indicated by* × *and arrow. Circles represent results from analytical method 1, squares by analytical method 2.*

mycin, and tetracycline. Although the change occurred with lower concentrations (10 µg/ml) of chloramphenicol where lysis was not obviously a problem, the phase density of the rounder-shaped cells was reduced. It was important to show whether or not the amount of wall teichoic acid had altered during incubation at 45°C in the presence of chloramphenicol. Walls were, therefore, prepared from cells of 172ts 200B that had been incubated for 1 h in the presence of the antibiotic at 45°C after being grown at 30°C in its absence. These walls were compared with those from cells first incubated at 30°C followed by 1 h at 45°C in the absence of chloramphenicol. Total phosphorus, glycerol, and galactosamine in the walls from cells incubated at 45°C in the absence of antibiotic were reduced as expected. This was not so in the presence of the antibiotic, except for galactosamine where some slight (8 to 10%) fall may have occurred. Therefore, although there was some morphological change, none of the other phenotypic changes could be detected.

DISCUSSION

Both double mutants of *B. subtilis* (strains 230 and RB973) that were grossly deficient in autolytic activity as well as being *rodA* in genotype still showed increase in wall peptidoglycan, loss (partly by dilution) of wall teichoic acid, and cellular morphological change. The only observed difference from the fully autolytic *rodA*

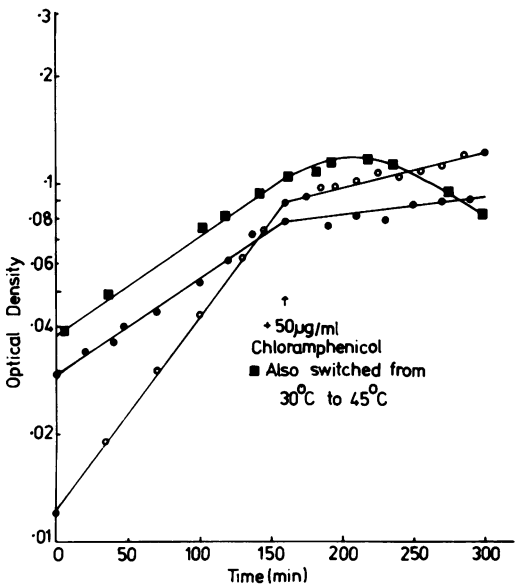

FIG. 4. *Effect of the presence of 50 μg of chloramphenicol per ml upon lysis when the growth temperature was changed from 30 to 45°C. Growth temperature:* (○) *30°C;* (●) *45°C;* (■) *changed from 30 to 45°C at time zero. The strain used was the autolytic-deficient strain RB973.*

strain 172ts 200B was that the cells in both the rod and coccal forms of the *lyt* mutant failed to separate. In the autolytic strain, the rods grew separately, whereas the cocci grew as clumps. These results do not support the suggestion (27) that the biochemical phenotype of the mutants is directly related to their loss of autolytic activity when grown at 45°C in the coccal form. It may be noted that autolytic-deficient strains also show little or no turnover of their walls (7, 18; R. S. Buxton, unpublished data). Both of the *rodA lyt* mutants, whether grown at 30 or 45°C, were about 5 to 10% as active in autolytic activity as 172*ts rodA* grown at 30°C or the wild-type strain 172 grown at either temperature. These results are for the autolysis of isolated walls and the action of cell extracts on SDS-treated walls at pH 9.5. At this pH the *N*-acetylmuramyl-L-alanine amidase is principally effective. Attempts to measure the minor β-*N*-acetylglucosaminidase autolysin were frustrated by the low activity of the enzyme after growth at either 30 or 45°C. It thus seems unlikely that the increases in proportion of cellular peptidoglycan during growth at 45°C are related to a loss of autolytic activity. The results do not rule out some role for small remaining amounts of autolytic enzyme in causing the morphological phenotype, and it is still possible that the remaining 5 to 10% of activity in the double mutants is essential. The failure of the cells to separate at either 30 or 45°C demonstrates that this function of the autolysins is not necessary for reversible conversion of rods to cocci, although detailed examination by quantitative electron microscopy (6, 11, 12) may show subtle differences in morphogenesis of the *rodA lyt* double mutant compared with the *rodA* mutant. The autolytic-deficient strain RB973, which is phosphoglucomutase negative, has a higher content of the unique peptidoglycan constituent, 2,6-diaminopimelic acid, than its parent wild type also grown at 30°C. The autolytic-deficient strain 230, which has phosphoglucomutase, has the same content of diaminopimelic acid as the parent strain. It would thus appear that the amount of wall substance in *B. subtilis* does not depend in any simple manner on the activity of the autolytic enzymes. It may be significant that the phosphoglucomutase-negative strain had modified walls as a result of its inability to make glycosylated polymers. These walls are very poor substrates for the principal autolytic enzyme. The walls of strain 230 are normal in this respect (7).

The effect of inhibiting protein synthesis on the increase in cellular peptidoglycan that occurs when the growth temperature is raised to 45°C

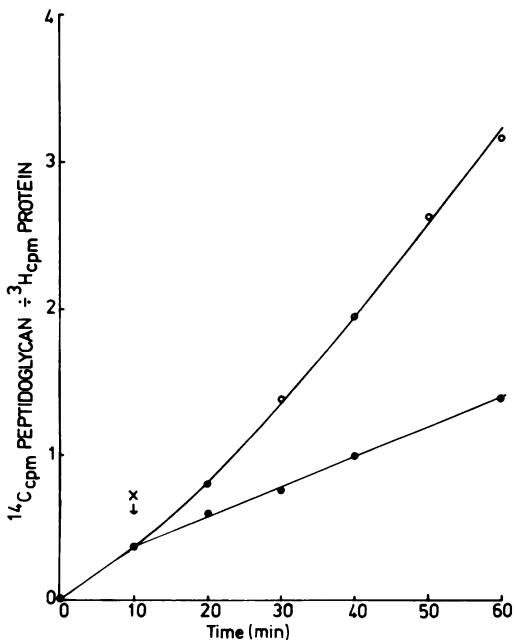

FIG. 5. *Peptidoglycan synthesis by strain 172ts 200B in the presence* (●) *or absence* (○) *of 10 μg of chloramphenicol per ml after changing the incubation temperature from 30 to 45°C at the time marked by* × *and arrow.*

supports the idea (23) that a derepression phenomenon is involved. Protein synthesis is a necessary concomitant, suggesting that the increased rate of synthesis depends upon either the formation of more active enzymes or the destruction of heat-labile inhibitors. Cell-free preparations made from *rodA* mutants grown at 45°C are more active in peptidoglycan synthesis than those from organisms grown at 30°C (M. V. Hayes, J. B. Ward, and H. J. Rogers, unpublished data).

The *rodA* mutants have a very complex phenotype, and the disturbances occurring when their growth temperature is raised are: (i) a gross change in shape; (ii) an increase in peptidoglycan; (iii) a reduction in glycerol teichoic acid; (iv) a cessation of formation of the *N*-acetylgalactosamine-glucose-phosphate polymer, due to a gross depletion of the necessary biosynthetic enzyme (10); and (v) a gross reduction in the activity of the autolytic enzymes. Yet no evidence has so far been forthcoming that these strains of *B. subtilis* are genetically complex. The above properties are to be compared with those of the genetically distinct *rodB* mutants, which show little change in their walls correlated with morphology and have normal autolytic activity, but have complex temperature-sensitive cation and anion requirements for the morphological change as well as for rapid growth (21, 22). The interrelations between the reduced amounts of teichoic acid and coccal morphology in the *rodA* mutants, taken together with the similar morphological effect of phosphate limitation on phosphoglucomutase-deficient strains (8, 9), suggest that a reduction in negative charge in the walls of bacilli is related to the rod-to-sphere change. Such a reduction could have some effect on the local availability of divalent cations, in particular Mg^{2+}, to the membrane surface. This may link the explanations for the *rodA* to those for the *rodB* genotype. The loss of autolytic activity that occurs in the *rodA* mutants may be related to the similar loss by phosphoglucomutase-deficient mutants (8, 9), one of which has been used here, as well by other species organisms (13, 25) that have modified teichoic acids in their walls and which become resistant to the autolysins. It would appear that, for active autolytic enzymes to be formed, the wall must be a satisfactory substrate.

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