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Lipoteichoic Acid from *Bacillus subtilis* subsp. *niger* WM: Isolation and Effects on Cell Wall Autolysis and Turnover

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Lipoteichoic acid (LTA) was extracted by means of hot aqueous phenol from *Bacillus subtilis* subsp. *niger* WM cells grown under various conditions in chemostat culture. The extracts were partially purified by nuclease treatment and gel permeation chromatography. Chemical analyses revealed a composition consistent with a polyglycerol phosphate polymer. The influence on autolysis of the LTAs thus obtained was studied with both whole cells and autolysin-containing native walls of *B. subtilis* subsp. *niger* WM. Lysis rates of phosphate-limited cells could be reduced to about 40% of the control rate by the addition of LTA, whereas lysis of cells grown under phosphate-sufficient conditions was affected to a much lesser extent. The lysis of native walls prepared from variously grown cells proved to be fairly insensitive to the addition of LTA. The effect of LTA on wall turnover was studied by following the release of radioactively labeled wall material during exponential growth. The most obvious effect of LTA was a lowered first-order rate of release of labeled wall material; calculations according to the model for cell wall turnover in *Bacillus* spp. formulated by De Boer et al. (W. R. De Boer, F. J. Kruyssen, and J. T. M. Wouters, J. Bacteriol. 145:50–60, 1981) revealed changes in wall geometry and not in turnover rate in the presence of LTA.

The widespread and phenotypically invariant occurrence of lipoteichoic acids (LTAs) and related substances in the membranes of many gram-positive bacteria (31, 38, 46) indicates a physiological function for these compounds that is indispensable for growth of these strains. The studies so far performed have focused on the role of LTA as membrane carrier in the synthesis of wall teichoic acids (21, 29), as regulator (inhibitor) of the autolytic activity in several gram-positive strains (12, 13, 23, 40), or as scavenger of bivalent cations (31), or on a role in the adherence of oral streptococci to dental surfaces (11). The investigations reported here were aimed to elucidate the possible role of LTA from *Bacillus subtilis* in the in vivo regulation of its autolysins.

During exponential growth of *B. subtilis* (1, 15, 33, 36) and other microorganisms (5, 6, 8, 25), the activity of these wall-bound enzymes becomes manifest as cell wall turnover: fragments of the wall (peptidoglycan and, when present, covalently attached anionic polymers [5, 9, 16, 32]) are released into the extracellular fluid by hydrolysis of bonds in peptidoglycan (9, 32). Under conditions unfavorable for growth and anabolism (e.g., during inhibition of wall synthesis by suitable antibiotics [18] or incubation in the absence of a carbon source [18]), the autolytic activity may lead to dissolution of the wall. It is known that LTAs and other membrane components, such as cardiolipin (CL), are able to prevent or at least inhibit lysis under these conditions (12, 13, 23, 40). Based on these findings, it has been proposed frequently that these amphiphiles also play a role in the in vivo regulation of autolytic activity (13, 15). Also, their location at the wall-membrane junction (38) renders them likely candidates for this important function.

To the best of our knowledge, no attempts have been made to relate data on the effect of LTA or CL on in vitro autolysis to the effects of these compounds on the autolytic activity in situ. Strains exhibiting cell wall turnover are suitable objects for such investigations, since the influence of LTA on wall turnover can easily be measured. Therefore, LTA was isolated from *B. subtilis* subsp. *niger* WM, and its effect on lysis of whole cells, of autolysin-containing native walls, and on cell wall turnover was investigated. The effects were compared with those obtained in similar experiments with (beef heart) CL to gain insight into the role of these compounds in the in vivo regulation of autolytic enzymes. As a corollary, we established the presence of LTA and its composition in cells grown under different conditions in chemostat culture.

(These results were taken from a thesis submitted in fulfillment of the requirements for the Ph.D. degree by P.D. Meyer to the University of Amsterdam, Amsterdam, The Netherlands.)

MATERIALS AND METHODS

Strain. *B. subtilis* subsp. *niger* WM, as described previously (30), was used throughout.

Media and growth conditions. Batch cultivation was performed in nutrient broth (Difco Laboratories, Detroit, Mich.) or in enriched minimal medium as detailed in reference 18. The composition of the latter medium is based on the recipe of Evans et al. (20) with all nutrients in nonlimiting concentration, with 100 mM sodium glutamate instead of ammonium chloride, and enriched with 0.2% (wt/vol) yeast extract (Difco Laboratories). Growth was followed turbidimetrically at 540 nm (optical density at 540 nm [OD_{540}]), and the OD readings were corrected for deviations from Beer's law (AOD; 43). AOD of 1,000 corresponded to a bacterial dry weight of 240 $\mu g\ ml^{-1}$. Chemostat cultivation under potassium, magnesium, glucose, or phosphate limitation was performed as described previously (30).

Sampling. Cells to be used for autolysis experiments were harvested by centrifugation (10 min at 4°C and 12,000 × *g*), washed once by suspension in ice-cold demineralized water, and centrifuged again as above. The washed cells were suspended in the desired buffer (see below). Autolytically

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active walls were prepared from cells harvested from the overflow tube of the culture vessel: the overflow was collected in an ice-cooled receiver as long as was needed to obtain the desired dry weight of cells, but never longer than 4 h to minimize autolysis of the collected cells. Cells thus harvested were washed as described above and stored as a frozen suspension at −20°C. Steady-state cells used for the extraction of LTA were harvested by a heat trap procedure as detailed in reference 30, washed as described above, and freeze-dried.

Extraction of LTA. LTA was extracted from whole cells by the hot aqueous phenol procedure described by Thaniyavarn et al. (42). The extracts were partially purified by RNase and DNase treatment and gel permeation chromatography on Sepharose 6B (Pharmacia, Uppsala, Sweden) as detailed in references 14 and 42).

Deacylation of LTA. Deacylation of LTA was performed with methanolic KOH by the procedure given by Button and Hemmings (7).

Preparation of native walls. Native walls were prepared by suspension of about 1.5 g (dry weight) of cells in 30 ml of ice-cold demineralized water. The suspension was transferred to a 60-ml glass bottle containing 20 ml of Ballotini beads (diameter, 0.1 mm) and 2 drops of polypropyleneglycol (Fluka AG, Buchs, Switzerland). The bottles were shaken in a Braun MSK homogenizer at maximum speed for 4× 2.5 min under constant cooling with liquid CO_2. Glass beads were removed by filtration over a sintered-glass filter (G3), and whole cells and other heavy debris were removed by a slow centrifugation run (10 min at 1,000 × *g* and 4°C). A wall pellet was then obtained by centrifuging the supernatant for 20 min at 12,000 × *g* and 4°C. Walls were washed three times with ice-cold demineralized water by suspension and centrifugation (20 min at 12,000 × *g* and 4°C) and finally stored as a frozen suspension with a dry weight of about 5 mg of wall dry weight ml^{-1}.

Turnover experiments. Cells were labeled pulsewise or continuously in their walls during exponential growth in batch or chemostat culture by using *N*-acetyl-D-[^{3}H]glucosamine ([^{3}H]GlcNAc). After the labeling period, the remaining radioactive substrate was removed by a centrifugation procedure (15). Cells labeled in batch cultures were suspended in the same medium at an OD_{540} of about 0.2 and reincubated. To parallel flasks containing a sample of the same cell suspension as the growing control culture, LTA or beef heart CL (Sigma Chemical Co., St. Louis, Mo.) was added in concentrations and at times specified below. Cells labeled in chemostat culture were suspended after centrifugation in prewarmed minimal medium as used in the chemostat buffered with 50 mM imidazole-HCl at pH 7.0 and lacking the limiting nutrient. This suspension was divided over several flasks containing the same medium supplemented with the limiting nutrient in nonlimiting concentrations at an OD_{540} of about 0.2. One flask received no further additions, but to the others LTA or CL was added in concentrations and at the times given below. Wall turnover was measured in the cultures by following the decrease of cell-bound radioactivity as described elsewhere (15). The wall turnover parameters were calculated as described by De Boer et al. (15). The decrease of cellular label per milliliter of culture exhibits first-order kinetics after a lag time (Θ, in hours). The rate constant of this decrease (λ) is expressed per hour. The fraction of the total wall material lost per hour is called κ (h^{-1}), while *k* is the percentage of wall lost per generation time. The generation time follows from the specific growth rate (μ, h^{-1}) which is equal to the dilution rate in chemostat cultures or can be calculated from the increase in AOD with time. X_B represents the percentage of wall material susceptible to the hydrolytic activity of the autolysins. For the derivation of these parameters, refer to De Boer et al. (15).

Lysis assays. Lysis of whole cells or native walls was followed by the decrease of the OD_{540} during incubation at 37°C and pH 9.0 (50 mM glycine-NaOH) or at pH 6.0 (50 mM succinate-NaOH). In the case of experiments with native walls, the buffers also contained 20 mM $MgCl_2$. The influence on lysis of walls or cells by LTA or CL was investigated by the addition of the compound of interest to the desired concentration from concentrated stock solutions. The control of the CL experiments contained ethanol (the solvent of the phospholipid) to the highest concentration applied with CL. Occasionally, lysis of whole cells also was determined from the release of a radioactive wall label ([^{3}H]GlcNAc) during incubation at pH 9.0 (18).

Analytical procedures. Phosphorus was determined according to Chen et al. (10) after oxidation of the dried sample with 70% $HClO_4$ as described by Kruyssen et al. (30). Fatty acid esters were assessed by the procedure of Snyder and Stephens (39), with methylstearate as the standard. The determinations of glycerol, total hexose, glucuronic acid, hexosamines, diaminopimelic acid, protein, DNA, and RNA are detailed elsewhere (16, 30). Glycerol, amino sugars, and amino acids were assayed after 6 M HCl hydrolysis (30). For paper chromatography, the LTA was hydrolysed in 4 M HCl for 4 h at 100°C. Alkaline phosphatase treatment was performed as described previously (17).

Chromatographic procedures. Gel permeation chromatography for the purification of LTA was performed with Sepharose 6B (Pharmacia). Column dimensions were 50 by 2.5 cm, and upward elution took place with 0.2 M ammonium acetate buffer (pH 6.9)–0.01% (wt/vol) NaN_3, which also was used for equilibration of the gel. Fractions of 3 ml were collected at a flow rate of 15 ml h^{-1}, and each second fraction was assayed for phosphorus as described before. The extinction at 260 nm (A_{260}) was monitored continuously with a flowthrough cell (Uvicord; LKB-Produkter AB, Bromma, Sweden). The void volume (V_0) was determined with blue dextran 2000 (Pharmacia).

Descending paper chromatography of acid or alkaline hydrolysates of LTA with or without alkaline phosphatase treatment was performed on Whatman no. 3 paper with propanol-1–NH_3 (specific gravity, 0.89 g $liter^{-1}$)–H_2O (6:3:1, vol/vol/vol) as eluant. Hydrolysates were applied as streaks, with the following compounds as standard: glucose, glycerol, glycerol-1-phosphate, and 1-*O*-β-glucosylglycerol (obtained from W. R. De Boer). The chromatogram was run for approximately 16 h. After drying, the following sprays were applied for the detection of the hydrolysis products: alkaline $AgNO_3$ treatment for carbohydrates (44), periodate-Schiff reagent for vicinal glycol groups (4), and an $FeCl_3$-acetylsulfonic acid procedure for phosphate esters (45).

RESULTS

Isolation of LTA. Elution of the nuclease-treated hot phenol extracts of whole cells of *B. subtilis* subsp. *niger* WM on Sepharose 6B resulted in the profiles shown in Fig. 1 (cf. reference 42). The peak with the lowest apparent molecular weight (eluting around fraction 80) probably contained mostly nucleic acids, as judged by its high A_{260}, and was not investigated further. In the area between the most conspicuous peaks, some phosphate-containing material also was

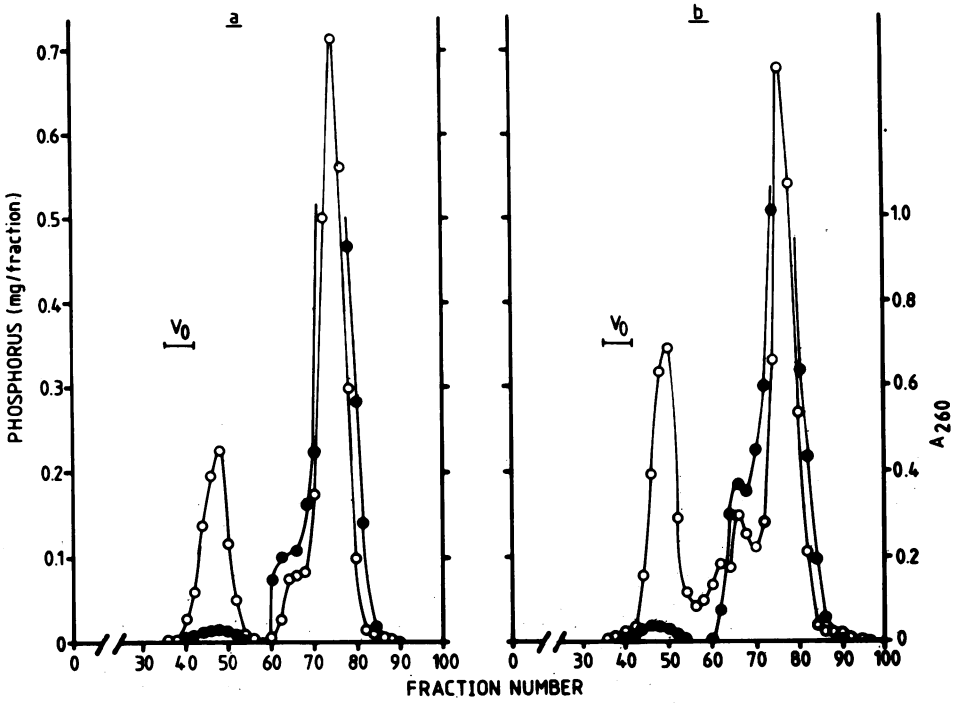

FIG. 1. Chromatography of crude LTA preparations of *B. subtilis* subsp. *niger* WM on Sepharose 6B. Phosphate (a)- or potassium (b)-limited bacteria were extracted with hot aqueous phenol; the extracts were treated with RNase and DNase and, after removal of the enzymes by a second phenol treatment, were chromatographed on Sepharose 6B columns (2.5 by 50 cm), using 0.2 M ammonium-acetate (pH 6.9) as the eluant. Fractions of 3 ml were collected, and their phosphorus content (○) and A_{260} (●) were determined as described in the text. V_0, Void volume.

present, which probably represents wall teichoic acid and deacylated LTA. The presence of wall teichoic acid can be inferred from the fact that this peak was more pronounced after extracting cells grown under phosphate-sufficient conditions (Fig. 1b) rather than cells grown under a phosphate limitation (Fig. 1a). The presence of deacylated LTA was deduced from the elution profile of chemically deacylated LTA, which eluted around fraction 70 (data not shown). Since it is known that LTA readily forms micelles due to its amphipathic nature, and thus shows a high apparent molecular weight in gel permeation chromatography as applied here, the phosphorus-containing fractions eluting directly after the void volume were pooled, freeze-dried, and weighed. The yield of LTAs thus obtained generally was about 5 mg g of cellular dry weight^{-1}, a value comparable to that reported elsewhere (27). Only under phosphate limitation did the yield of LTA seem somewhat lower, being about 2 mg g of cellular dry weight^{-1}. The reduced LTA content of phosphate-limited cells already could be inferred from the height of the LTA peak in the final purification step on Sepharose 6B (Fig. 1a and b).

Chemical composition of LTA. LTAs thus obtained from variously limited cells (all grown at a dilution rate of about 0.2 h^{-1}) were chemically analyzed. Data of these analyses are compiled in Table 1. It can be concluded that these extracts contained phosphorus and glycerol in almost equimolar amounts, as might be expected from a teichoic acid. Lipid ester determinations of LTA isolated from magnesium- or potassium-limited cells showed values close to those encountered in LTAs of *B. subtilis* W23 (14) and *B. licheniformis* (7). Only in the LTA derived from phosphate-limited cells was a relatively high lipid ester content found.

With respect to both the hexose and glucosamine content, no large differences were noticeable between the LTAs isolated from differently limited cells. The low molar ratio between hexose and phosphorus indicates a low degree of hexose substitution of the glycerol phosphate chain of LTA compared with the virtually complete hexose substitution of the wall teichoic acid (17).

Nucleic acid and protein contamination of the various LTAs was low and similar to the amounts reported in other investigations of *B. subtilis* LTA (14, 42). Analyses of components of peptidoglycan (i.e., diaminopimelic acid) or teichuronic acid (i.e., glucuronic acid, and only on the extracts from phosphate-limited cells) revealed that no detectable amounts of these wall constituents were present in our LTA preparations (data not shown).

TABLE 1. Chemical composition of LTA of *B. subtilis* subsp. *niger* WM

Component	Limitation[a]					
	P		Mg		K	
	μg mg^{-1}	MR[b]	μg mg^{-1}	MR	μg mg^{-1}	MR
Phosphorus	65	1.00	74	1.00	72	1.00
Glycerol	166	0.86	193	0.88	185	0.86
Hexose	94	0.25	81	0.19	84	0.20
Glucosamine	11	0.03	13	0.03	15	0.04
Fatty acid ester	127[c]	0.23	96[c]	0.15	75[c]	0.12
Protein	96	—	95	—	ND	—
Nucleic acids	23	—	16	—	ND	—

[a] Bacteria were grown under phosphate (P), magnesium (Mg), or potassium (K) limitation (dilution rate, ca. 0.2 h^{-1}). ND, Not determined. —, Not applicable.

[b] MR, Molar ratio relative to phosphorus.

[c] Methylstearate as standard.

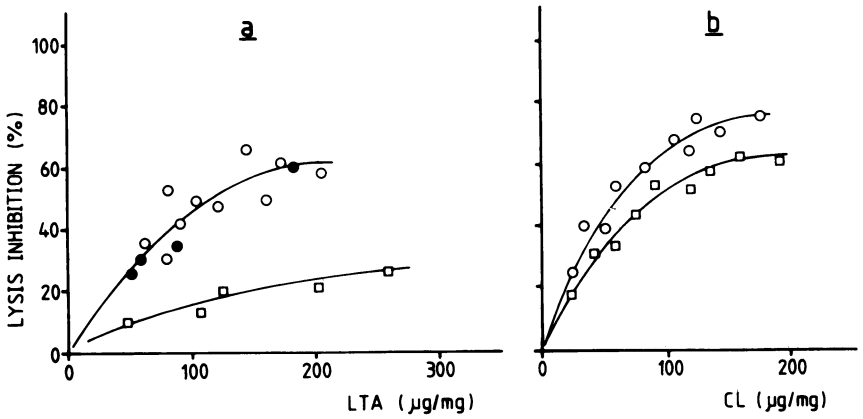

FIG. 2. Relationship between LTA (a) and CL (b) concentration and lysis inhibition of whole cells of *B. subtilis* subsp. *niger* WM. Water-washed cells from phosphate (○,●; μ, 0.25 h^{-1})- or glucose (□; μ, 0.27 h^{-1})-limited cultures were assayed for lysis at pH 9.0 (50 mM glycine-NaOH) and 37°C. Lysis rates were obtained from the decrease in OD_{540}, and the percentage of lysis inhibition was calculated from comparison of the lysis rate of the control suspension containing no LTA or CL with that of a suspension to which LTA or CL was added. LTA was derived from magnesium (open symbols)- or phosphate (closed symbols)-limited cells. Concentrations are expressed per milligram of bacterial dry weight.

Paper chromatography of the hydrolysis products obtained after alkaline or acid hydrolysis gave results consistent with the LTAs being polymers of glycerol phosphate. This component was readily detected and could be converted into glycerol by alkaline phosphatase treatment. The polymers were easily degraded during alkaline hydrolysis (in contrast to the wall teichoic acids from this strain [17]), which also indicates a low glucose substitution of the LTAs. The low level of sugar substitution was corroborated further by the fact that only low amounts of glucosylglycerol phosphates were found after acid hydrolysis. Among the products arising after alkaline hydrolysis, diglycerol triphosphate could be tentatively identified. Since this compound is diagnostic for a poly(1,3)-glycerol phosphate (3), the LTAs of *B. subtilis* subsp. *niger* WM also may have this structure.

Effect of LTA on autolysis. LTAs isolated as described previously were tested for their anti-autolytic properties with respect to both whole-cell lysis and lysis of native walls. Whole-cell lysis was measured at the optimum pH 9.0 (35), and from Fig. 2a it is clear that LTA isolated from phosphate-limited cells is as effective an inhibitor of whole-cell lysis as is that extracted from magnesium-limited cells. Furthermore, it is apparent that lysis of phosphate-limited cells is inhibited more strongly by LTA than lysis of cells grown under phosphate excess conditions. That the LTA preparations are devoid of D-alanine (22) probably does not affect the outcome of these experiments. An LTA extracted from cells harvested at 4°C and not purified by nuclease treatment to preserve the amino acid ester also exhibited lysis inhibition against phosphate-limited cells: with 150 μg of LTA mg of bacterial dry $weight^{-1}$, about 20% inhibition was obtained. A similar picture was obtained when effects of CL, which is also known for its lysis-inhibiting properties in various autolytic systems (13, 23, 40), were studied. With CL, however, the difference in lysis inhibition between phosphate- and glucose-limited cells was less clear than with LTA (Fig. 2b). Moreover, on a weight basis, CL proved to be a more effective inhibitor than LTA (cf. Fig. 2a and b). On a molar basis, however, the difference is less as CL has a lower molecular weight than LTA (e.g., reference 27). It should also be noted that fairly high concentrations of the amphiphiles had to be added to affect cellular lysis. Chemical deacylation of LTA resulted in the loss of the anti-autolytic properties of this compound (data not shown), as was already noted in similar experiments with LTAs from *Streptococcus faecium* (12) and *Staphylococcus aureus* (23). All data given above are based on OD readings to obtain cellular lysis rates. With cells radioactively labeled in their walls with [^{3}H]GlcNAc, it could be shown that lysis inhibition by LTA or CL, as judged by OD measurements, was accompanied by a lowered rate of release of wall label from the cells (data not shown).

The effect of homologous LTA and CL on the lysis of native walls was investigated with various wall preparations at pH 6.0 and 9.0. Thereby one can obtain information on amidase-effected lysis (at pH 9.0 [26, 28]) and on glucosaminidase (plus amidase)-effected lysis (at pH 6.0 [26, 28, 37]). It was found that cell walls derived from chemostat-grown cells containing teichoic acid, teichuronic acid, or both anionic polymers were hardly inhibited with respect to their lysis rates by LTA at pH 9.0 (Table 2). In fact, in some cases the lysis rate was slightly raised by the addition of LTA. Lysis of native walls prepared from batchwise-grown cells (grown at high growth rates) was somewhat inhibited by LTA at pH 9.0 (Fig. 3; Table 2). At the lower pH, LTA exerted an inhibitory influence on the lysis of native walls, but only if teichuronic acid was present as the anionic polymer (i.e., in the case of walls prepared from phosphate-limited cells) or if walls from fast-growing batch-cultured cells were used (Table 2). As was already noted with lysis of whole cells, CL seemed, on a weight basis, a more effective inhibitor than LTA (Fig. 3; Table 2), but on a molar basis this difference is less (see above). Moreover, the former amphiphile could inhibit the lysis of all wall preparations tested, irrespective of the type of anionic polymer present in the walls, and did so at both pH values (Table 2).

Effect of LTA and CL on a wall turnover. When cells labeled in their walls either pulsewise or continuously with [^{3}H]GlcNAc were chased in the presence of LTA, a lowered first-order rate constant λ of label release was observed, while the growth rate remained virtually unchanged (Fig. 4a and b). This effect proved to be independent of the growth rate, since it was observed with fast-growing batch cultures (Fig. 4a) and with cells taken from slowly growing chemostat cultures (Fig. 4b). Remarkably, only when LTA had been added at the start of the chase experiment was λ lowered

TABLE 2. Influence of homologous LTA and beef heart CL on lysis of native walls from variously grown *B. subtilis* subsp. *niger* WM

Growth conditions	LTA (μg ml^{-1})	CL (μg ml^{-1})	Lysis rate[a]			
			pH 9.0		pH 6.0	
			LTA	CL	LTA	CL
Potassium limitation (μ = 0.2 h^{-1})	0	0	1.00	1.00	1.00	1.00
	10		1.03		1.22	
	20	20	1.18	0.77	1.22	ND
	100	40	1.17	0.75	1.23	ND
	200	80	0.93	0.50	1.08	0.13
Phosphate limitation (μ = 0.2 h^{-1})	0	0	1.00	1.00	1.00	1.00
	10		0.95		ND	
	20	20	1.12	0.77	ND	ND
	100	40	1.15	0.65	0.78	ND
	200	80	1.06	0.51	0.55	0.33
Phosphate limitation (μ = 0.55 h^{-1})	0	0	1.00	1.00	1.00	1.00
	10		1.15		1.00	
	20	20	1.11	0.83	0.92	ND
	100	40	1.36	0.75	0.85	ND
	200	80	1.28	0.50	0.71	0.60
Batch (enriched minimal medium) (μ = 1.25 h^{-1})	0	0	1.00	1.00	1.00	1.00
	100		0.66		ND	
	200	80	ND	0.45	0.61	0.45

[a] Lysis rates are expressed relative to that of the control suspension. ND, Not determined.

(Fig. 4a). Calculations according to the model for wall turnover in *Bacillus* spp. formulated by De Boer et al. (15) revealed that in the presence of LTA the turnover-sensitive fraction of the wall, X_B, was increased, while the parameters related to the enzymatic activity (κ and k) remained unchanged. It could also be shown that these effects of LTA were dependent on the ratio of LTA/bacterial dry weight (Fig. 5a). Deacylated LTA, wall teichoic acid, or teichuronic acid proved to be without any effect on growth rate or turnover parameters (data not shown).

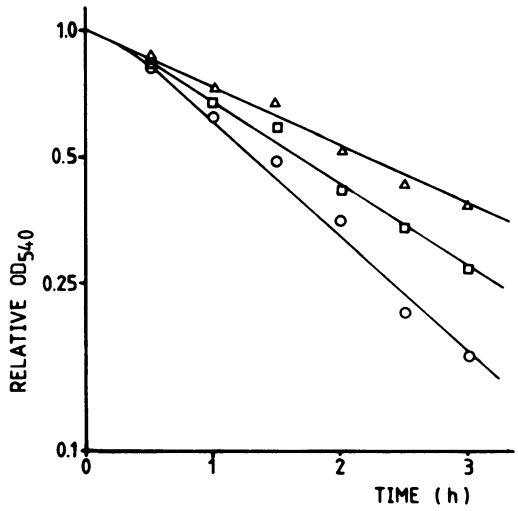

FIG. 3. Effect on lysis of native walls from *B. subtilis* subsp. *niger* WM of LTA (100 μg ml^{-1}; □) or CL (80 μg ml^{-1}; △). Walls were prepared from cells grown batchwise in enriched minimal medium (μ, 1.25 h^{-1}), and lysis was determined as a decrease in OD_{540} (expressed relative to the zero time value) at pH 9.0 (50 mM glycine-NaOH, 20 mM $MgCl_2$) and 37°C. The control experiment (○) contained no LTA or CL.

The effect of CL or, more precisely, that of a combination of ethanol and CL was more complicated, since in the presence of these compounds the growth rate of fast-growing batch cultures was lowered (Fig. 4c). Cells growing at submaximal rates (derived from chemostat cultures) were much more sensitive to the addition of these compounds; no exponential growth was observed for a prolonged period of time when these cells were chased in the presence of ethanol and CL (data not shown). The results given below are therefore only applicable to fast-growing batch cultures. CL dissolved in ethanol had an effect on wall turnover similar to that of LTA: addition of the phospholipid resulted in a decrease of the first-order rate constant λ (Fig. 4c). This phenomenon was independent of the moment of addition, contrary to the effect of LTA (Fig. 4a and d). As was found with LTA, CL did not influence the turnover rate (expressed by the parameter k), but the turnover-sensitive fraction of the wall, X_B, did increase in the presence of CL (Fig. 5b).

DISCUSSION

From the data presented above we may conclude that, also in *B. subtilis* subsp. *niger* WM, the presence of LTA is not subject to phenotypic variation to the same extent as the wall teichoic acid, the latter being virtually absent under phosphate limitation at low growth rates (30). Only a twofold reduction of the cellular LTA content was noted under these conditions, though a more drastic decrease was noted in phosphate-limited *B. licheniformis* (D. Button, M. K. Choudry, and N. L. Hemmings, Proc. Soc. Gen. Microbiol. 2:45, 1975). As far as the composition is concerned, the LTA isolated from *B. subtilis* subsp. *niger* WM does not deviate significantly from LTAs derived from other *Bacillus* species (see references 7, 14, and 42). Some differences between wall and membrane teichoic acid became evident: the former is substituted almost completely by glucose (17), whereas in the latter this substituent is barely present (Table 1). Moreover, the main wall teichoic acid in *B. subtilis* subsp. *niger* WM is a 2.3-linked poly(glycerol phosphate) (17), while probably the LTA is formed from 1,3-linked glycerol phosphate residues. This latter structure seems to be a common trait of the hydrophilic chain of the LTAs of many different species (31, 46), whereas the structural diversity of wall teichoic acids is far greater (2, 3, 46). These differences probably reside in the different biosynthetic routes leading to the respective polymers. Wall teichoic acids are synthesized from CDPglycerol and UDPglucose (2, 46), while the glycerol phosphate residues of LTA in *B. subtilis* probably are derived from L-α-phosphatidylglycerol (19) as is found in other gram-positive microorganisms (24, 41).

The question of whether LTA or CL or both play a role in the regulation of the autolytic activity of *B. subtilis* subsp. *niger* WM remains difficult to answer on the basis of the results presented here. Especially with LTA, the effect on the autolysins of this strain is rather complex: whole-cell lysis is clearly inhibited (Fig. 2a), but lysis of native walls is barely influenced (Table 2). On the other hand, CL shows a much more general lysis-inhibitory behavior (Fig. 2b; Table 2). The differences in anti-autolytic properties of these compounds (or of the micelles formed by each) may be related to differences in molecular size and their subsequent penetration through the wall and to the accessibility of the autolysins within the wall. (Micelles are probably required for the anti-autolytic effects of these amphiphiles, since both

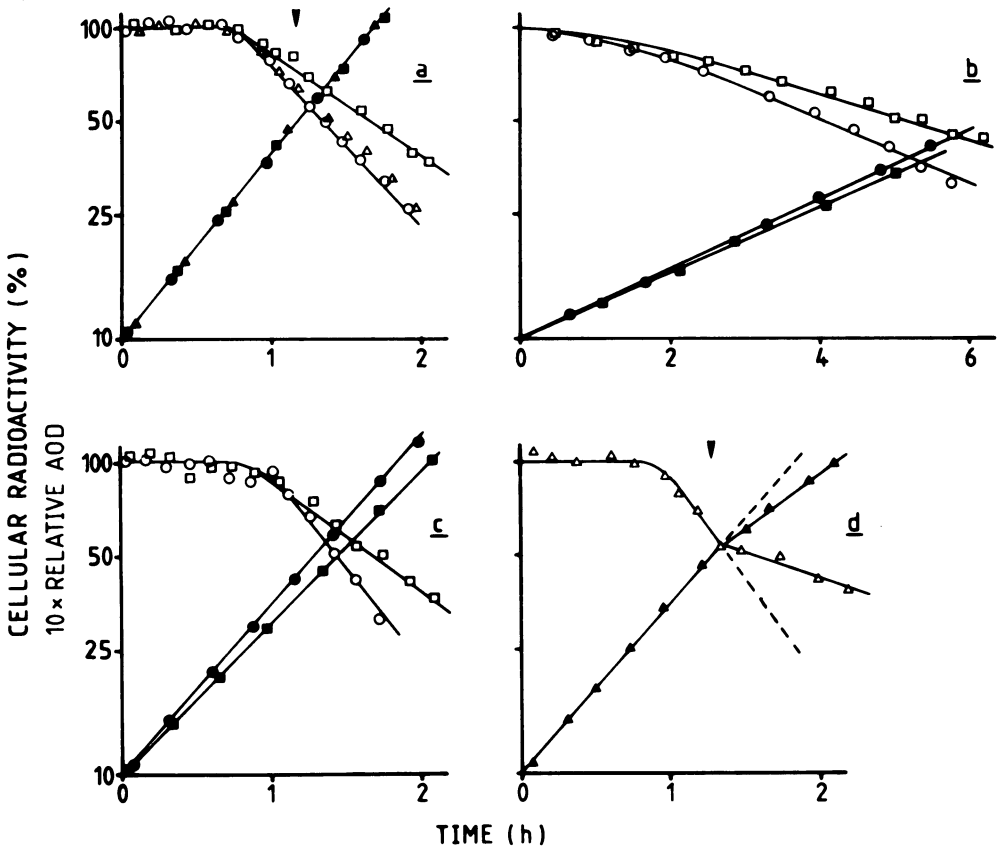

FIG. 4. Influence of LTA (a and b) or CL (c and d) on growth and cell wall turnover in *B. subtilis* subsp. *niger* WM. Bacteria were grown batchwise in enriched minimal medium (μ, about 1.3 h^{-1}) and labeled pulsewise with [^{3}H]GlcNAc (a, c, and d) or grown under phosphate limitation (μ, 0.23 h^{-1}) and labeled for six generations with [^{3}H]GlcNAc (b). After centrifugation, cells were suspended in enriched minimal medium (a, c, and d) or imidazole-buffered minimal medium (b). Cellular radioactivity (open symbols) and growth (closed symbols; expressed as AOD, relative to the zero time value) were determined as described in the text. Chasing took place in the absence (○,●) or presence (□,△, ■,▲) of LTA (a and b; 40 μg ml^{-1}) or CL (c; 40 μg ml^{-1}). The arrowheads in (a) and (d) indicate the time of addition of LTA (a; 160 μg ml^{-1}) or CL (d; 160 μg ml^{-1}) in the experiments represented by the triangles.

addition of Triton X-100 and deacylation of LTA prevent the effect on lysis of whole cells [data not shown, but see references 12, 13, and 23].)

The considerable loss of the lysis-inhibiting properties of LTA when measured with native walls instead of whole cells (Fig. 2a; Table 2) may be caused by the loss of a proper membrane (or wall) structure during the isolation of the walls. Lysis brought about by the amidase (i.e., measured at pH 9.0) is only inhibited when walls from fast-growing batchwise-cultured cells are used (Table 2), which may indicate a difference between the walls of batchwise- and chemostat-cultured cells. The nature of this difference is, however, unknown. These findings are in contrast to those of others (12), who could not find a lysis-inhibiting influence of homologous LTA with native walls of batch-cultured *B. subtilis* 168 cells. It may well be that these investigators applied concentrations of LTA that were too low, since we found that rather high concentrations had to be used to produce the observed effects. That at pH 6.0, at which both autolysins are active (unpublished observations, but see references 26 and 28), lysis of some types of walls is inhibited by LTA, together with the finding that the amidase is not affected by LTA, suggests that the other autolysin, the glucosaminidase, in these walls is subject to inhibition by LTA. This conclusion is only applicable, however, to teichuronic acid-containing walls (from phosphate-limited cells) or to walls from fast-growing, batch-cultured cells. Also, the data on the influence of LTA on the purified autolysins of *B. subtilis* 168 lend support to the conclusion that the glycan-splitting enzyme can be inhibited by LTA (37).

How far the absence of the D-alanine substituents of LTA (these will be removed during the nuclease treatment of the crude LTA preparations [22]) influences the outcome of these experiments remains unclear. With lysing walls from *Staphylococcus aureus* it has been found that native (that is, D-alanine-containing) LTA showed no lysis-inhibitory activity (22, 23), but recently Rogers et al. (37) showed that removal of the amino acid substituent from LTA of *B. subtilis* 168 had no effect on inhibition of the glucosaminidase from this strain. Furthermore, it should be noted that so far only with *Staphylococcus aureus* has an effect of the degree of alanylation on in vitro autolysis been reported. Recent data indicate that the growth of this organism is unaffected by the D-alanine content of its LTA (34), which suggests that the LTA functions in vivo are independent of the degree of alanylation. Finally, it should be mentioned that LTAs have been isolated that do not contain alanine ester (22). Hence, a generalization on the physiological role of the D-alanine content of LTAs cannot be made, and the effects found with the lytic system of *Staphylococcus aureus* do not necessarily apply to that of *B. subtilis*.

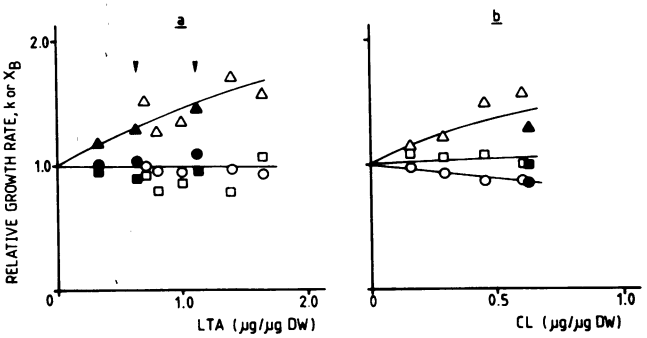

FIG. 5. Relationship between LTA (a) and CL (b) concentration and growth rate (○,●) and the turnover parameters k (□, ■) and X_B (△, ▲) (for definitions of the latter, see text). The data were obtained from experiments with pulse-labeled (open symbols) or continuously labeled (closed symbols) cells, and the values of growth rate, k, and X_B are given relative to those obtained in control experiments which contained no LTA or CL. The arrowheads in (a) denote experiments with cells from chemostat cultures; the other data are from experiments with fats-growing batch cultures. LTA and CL concentrations are given per microgram of cell dry weight (DW).

Lysis inhibition by CL clearly is independent of teichoic or teichuronic acid being present in the wall. Probably, this compound inhibits both autolysins, as inhibition of lysis of native walls is observed at both pH values (Table 2). As far as the glucosaminidase is concerned, Rogers et al. (37) reported that this enzyme is inhibited strongly by CL.

When one considers the effect of LTA and CL on the activity of the autolysins in situ (that is, on peptidoglycan turnover), it is clear that mainly a lowering of the first-order rate constant λ occurs (Fig. 4a and b). At first sight, this may be interpreted as indicating that wall turnover is inhibited under these conditions. In our opinion, however, this is only so if the lag time, Θ (the moment first-order release of label sets in), remains unaffected. From the model for wall turnover formulated by De Boer et al. (15), it follows that the geometry of the wall, or more precisely the occurrence of two fractions of the wall with different sensitivities to turnover, must be taken into account to make statements about the turnover rate. This is most easily appreciated from the equation used to calculate the turnover-sensitive fraction of the wall (X_B): $X_B = \kappa/\lambda$ or $\kappa = \lambda \cdot X_B$. In this formula, κ is the rate constant of the turnover process from which the parameter k, the percentage of wall lost per generation time, is obtained by multiplication with the generation time. It will be evident that in itself a lowered λ does not indicate a lowered κ and hence a lowered k, as X_B may have increased concomitantly with the decrease of λ. Under the influence of LTA or CL, this appears to be the case (Fig. 5a and b); apparently, *B. subtilis* subsp. *niger* WM can change its wall geometry to maintain the turnover rate at the same (necessary) level. As to how this is accomplished, one can but speculate. One may assume that LTA and CL also inhibit the autolytic activity in vivo and that for exponential growth to occur the turnover rate k must be maintained at the same level. An increase of X_B is then necessary to counteract this inhibitory effect. Alternatively, from the fact that LTA is active only when present at the start of a chase experiment (Fig. 4a), one may infer that this amphiphile interacts with the autolysins that are in the close vicinity of the membrane and affects that fraction of the wall which is immune to turnover in such a way that it decreases. This, in turn, causes an increase of the turnover-sensitive fraction of the wall. That CL also is active when added later in a chase may arise from differences in the respective structures of (the micelles of) these substances, as was already mentioned to explain their different behavior towards cellular and wall autolysis.

The question now arises as to whether LTA and CL do or do not play a role in the regulation of wall turnover in *B. subtilis*. From the data presented above no simple conclusions can be drawn, but a role in the establishment of the geometry of the wall with respect to the sensitivity to turnover may be inferred. The underlying mechanisms remain unknown, however, since an extrapolation from data obtained in in vitro experiments on lysis inhibition by these compounds to data from experiments with growing cells cannot easily be made.

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