A high cell wall negative charge is necessary for the growth of the alkaliphile *Bacillus lentus* C-125 at elevated pH

Rikizo Aono,¹ Masahiro Ito,¹ Keith N. Joblin² and Koki Horikoshi¹

Author for correspondence: Rikizo Aono. Fax: +81 45 924 5819.

¹ Department of Bioengineering, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Nagatsuta 4259, Yokohama 226, Japan

² Grasslands Research Centre, AgResearch Private Bag 11008, Palmerston North, New Zealand

The structural components in cell walls of three mutants of a facultative alkaliphile, *Bacillus lentus* C-125, defective in certain cell-wall components were characterized in detail. The cell walls of the wild-type C-125 were thick and increased in thickness when grown at high pH. Electron microscopy showed that triple layers developed when the bacteria were grown in an alkaline environment. In contrast, cell walls of teichurono-peptide (TUP)-defective mutants consisted of a single layer. For both the wild-type and mutants, the cell-wall concentrations of the acidic structural polymers teichuronic acid and TUP increased with respect to peptidoglycan as culture pH increased. For all four strains, the anion content of their cell walls was the greatest at high pH. The cell-wall density of the negatively charged compounds (uronic acids plus L-glutamic acid) was calculated as about 3 and 9 equivalents (1 cell wall region)⁻¹ for C-125 cells grown at pH 7 and 10, respectively. At high pH, the specific growth rates of the two TUP-defective mutants were much lower than those of the wild-type. It is concluded that increased levels of acidic polymers in the cell walls of alkaliphilic bacteria may be a necessary adaptation for growth at elevated pH.

Keywords: alkaliphile, *Bacillus lentus*, cell wall, negative charge

INTRODUCTION

The facultative alkaliphile *Bacillus lentus* C-125 grows between pH 6.8 and 10.8. This organism used to be called *Bacillus* sp. C-125 (Aono & Horikoshi, 1983); however, recently this organism was shown to be related to *B. lentus* (Aono, 1995). It has been reported that a $\Delta\psi$ -dependent Na^+/H^+ antiporter on the cell membrane helps to maintain the intracellular pH around neutrality (Kitada *et al.*, 1994). The bacterial cell wall also appears to be important for the development and maintenance of alkaliphily. Protoplasts prepared from strain C-125 are unstable at alkaline pH (Aono *et al.*, 1992) and regenerate at neutral pH but not at alkaline pH (Aono *et al.*, 1993). The cell wall of alkaliphiles possibly acts as an obstacle to the high extracellular concentrations of hydroxide ions and as a reservoir of hydrogen ions generated intracellularly by respiration.

The cell walls of *B. lentus* C-125 contain peptidoglycan,

teichuronic acid (TUA) and teichurono-peptide (TUP). The peptidoglycan is an A1 γ type that is identical to that in the cell walls of the neutrophilic *Bacillus subtilis* (Aono *et al.*, 1984). The TUA consists of galacturonic acid, glucuronic acid and N-acetyl-D-2-amino-2,6-dideoxygalactose (fucosamine) in a molar ratio of 1:1:1 (Aono & Uramoto, 1986). The TUP is a polymer in which polyglutamic acid binds covalently to polyglucuronic acid (PGU) and has a 1:4.5 molar ratio of glucuronic acid to L-glutamic acid (Aono, 1985, 1987, 1989). The quantities of acidic polymers in the cell walls of strain C-125 increase when cells are grown at alkaline pH (Aono & Horikoshi, 1983; Aono, 1985). Mutants defective in the acidic polymers grow poorly on an alkaline solid medium (Aono & Ohtani, 1990; Ito *et al.*, 1994).

These studies suggested that the acidic polymers in the cell walls might be important for bacterial growth in an alkaline environment. However, this conclusion was drawn from experiments with cells from cultures grown at initial pH values of 7.0 or 10.0 but lacking pH control during growth. Moreover, there have been few detailed measurements of the composition of the cell walls of alkaliphiles and little is known about the effects of culture

Abbreviations: DAP, diaminopimelic acid; PGU, polyglucuronic acid; TCA, trichloroacetic acid; TUA, teichuronic acid; TUP, teichurono-peptide.

pH on cell-wall morphology. We have examined cell-wall-defective mutants grown under pH control. In this paper, we describe in detail the pH-dependent alteration of the chemical composition of cell walls of the C-125 wild-type and the mutants, the pH-dependent specific growth rates of the strains, and the ultrastructure of cell walls of the strains grown at several pH values.

METHODS

Bacterial strains. The strains used in this study are listed in Table 1. All mutant strains are derivatives of an alkaliphilic strain of *B. lentus* C-125 (FERM 7344; Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba, Japan). Strain C-125-11, a mutant lacking TUA, was obtained by treating a threonine-requiring and streptomycin-resistant derivative of C-125 with ethylmethanesulphonate (Aono & Ohtani, 1990). Strain C-125-90 obtained from C-125-11 lacks TUA and the polyglutamic acid moiety of TUP. Strain C-125-F19 (methionine-requiring and nalidixic-acid resistant) was constructed by protoplast fusion between C-125-90 and the wild-type strain. The cell walls of C-125-F19 contain TUA and lack the polyglutamic-acid chain of TUP (Aono *et al.*, 1994; Ito *et al.*, 1994).

Media and growth conditions. The bacteria were aerobically grown at 37 °C in a medium consisting of (l deionized water)⁻¹: K₂HPO₄, 13.7 g; KH₂PO₄, 5.9 g; citric acid, 0.34 g; MgSO₄·7H₂O, 0.05 g; glucose, 5 g; peptone, 5 g; yeast extract, 1 g; Na₂CO₃, 10.6 g. The pH of this medium was about 10 (Aono, 1985).

When it was necessary to keep the pH constant during growth, bacteria were grown in 3 l batches in a 10 l jar equipped with a pH-stat apparatus. The required pH values were initially established in the medium described above by using the following buffers and then maintained by the automatic addition of 1 M HCl or 1 M NaOH during cultivation. The initial pH values were established by adjusting the medium with the following buffers: pH 6.5–7.0, 0.1 M K₂HPO₄/0.2 M NaCl/HCl; pH 8.0, 0.1 M K₂HPO₄/0.14 M NaCl/0.03 M Na₂CO₃; pH 9.0, 0.1 M K₂HPO₄/0.1 M NaHCO₃/0.05 M Na₂CO₃; pH 10.0–11.0, 0.2 M KCl/0.05 M Na₂CO₃/NaOH. Air was bubbled into the medium at a flow rate of 4.5 l min⁻¹. The culture was stirred at 350 r.p.m.

Preparation of the cell walls. Cells were harvested by centrifugation (8000 g, 4 °C, 10 min) of a portion of the culture containing cells at the late-exponential phase of growth. The cells (wet wt 7–10 g) were immediately suspended in 300 ml hot 2% (w/v) SDS solution containing 0.1 M NaCl, and autolytic enzymes (Aono & Sanada, 1994) were inactivated by incubation of the suspension at 80 °C for 30 min. Cells were collected by centrifugation (8000 g, 20 °C, 15 min), suspended in three times the volume of 0.1 M NaCl containing 0.1% NaN₃, and disrupted with a sonic oscillator (200 W, room temperature, 20 min). The sonicated lysate was centrifuged (8000 g, 20 °C, 15 min) to remove unbroken cells, and an equal volume of 4% (w/v) SDS solution was added to the broken-cell suspension. Mixtures were incubated at 80 °C for 30 min. Cell walls were sedimented by centrifugation (20000 g, 20 °C, 30 min) and then washed four times with 0.1 M NaCl containing 0.1% NaN₃. Washed cell walls were resuspended in 50 mM Tris/HCl (pH 7.9) containing 10 mM CaCl₂, and digested with trypsin (0.5 mg ml⁻¹) at 37 °C overnight. Cell walls were subsequently washed several times with 10 mM EDTA/NaOH (pH 7.3), 0.1 M NaCl/0.1% NaN₃, and deionized water (Aono & Horikoshi, 1983).

Isolation of non-peptidoglycan components from cell walls.

Cell walls were extracted with warm 5% (w/v) trichloroacetic acid (TCA) three times as described previously by Aono (1985). TCA extracts were exhaustively dialysed against running water in a dialysis tube with an *M_r* cut-off of 3500 (Spectropore). The dialysate was mixed with an equal volume of 0.1 M acetic acid/NaOH buffer (pH 5.0) and loaded on to a column of DE-52 (1.5 × 50 cm; Whatman) which had been equilibrated with 50 mM acetic acid/NaOH buffer (pH 5.0). The column was washed with 60 ml of the buffer and eluted with 60 ml of the same buffer containing 0.2 M NaCl, followed by a linear gradient elution from 0.2 to 0.6 M NaCl in the buffer (200 ml) at a flow rate of 80 ml h⁻¹. The column was finally eluted with buffer containing 0.6 M NaCl. Fractions (3 ml) were assayed for uronic acids, amino sugars and L-glutamic acid. The fractions containing these compounds were pooled and dialysed against deionized water (*M_r* cut-off 3500).

A portion of the samples was concentrated to about 1 ml in a rotary evaporator at 40 °C. The residual solution was applied to a column of Toyopearl HW55S (1.5 × 97 cm; Toyo Soda) (sold under the name of Fractogel TSK-HW55S in some areas) which had been equilibrated with 0.2 M NaCl. The column was eluted with 0.2 M NaCl. Fractions (1.3 ml) containing uronic acids were dialysed against deionized water.

***M_r* values.** *M_r* values of samples were estimated by gel chromatography on two columns of Shodex WS 802.5F (Showa Denko) connected in series to an HPLC apparatus. The columns were eluted with 0.1 M NaH₂PO₄/NaOH buffer (pH 7.2) at a flow rate of 1.0 ml h⁻¹. The elution pattern was monitored by measuring refractive index with a differential refractive index detector (RID-300; Nippon Bunko). *M_r* standards of authentic pullulans (Shodex Standard P-82; Showa Denko) were used to calibrate the gel chromatography.

TLC. Ascending Avicel cellulose thin-layer chromatograms were run using the following solvents: (A) ethyl acetate/pyridine/water/acetic acid (5:5:3:1, by vol.) at room temperature; (B) phenol/1% aqueous ammonia (5:2, v/v) at 43 °C. Separated components were located with alkaline silver nitrate (Trevelyan *et al.*, 1950) or ninhydrin (Stepka, 1957) spray. Glucosamine-HCl, glutamic acid, glucuronic acid and galacturonic acid (Wako Pure Chemical Industries) were used as the authentic standards.

Paper electrophoresis. Samples were spotted in the middle of Whatman no. 1 filter paper, 40 cm in length, and electrophoresed in 10% (v/v) acetic acid/pyridine buffer (pH 2.5) at 12.5 V cm⁻¹ for 2 h. Separated compounds were located with the alkaline silver nitrate or ninhydrin spray.

Analyses

(i) **Uronic acids.** Uronic acids were determined by the carbazole reagent method (Davidson, 1966) using glucuronic acid as a reference standard.

(ii) **Amino compounds.** Samples were hydrolysed in 4 M HCl for 16 h at 100 °C in sealed tubes. Amino sugars were assayed by the Elson–Morgan reaction (Wheat, 1966) using glucosamine or fucosamine as a reference standard. Authentic fucosamine was prepared from the walls of *B. lentus* C-125 (Aono & Uramoto, 1986). L-Glutamic acid content was determined by using L-glutamic acid dehydrogenase and diaphorase (Beutler & Michal, 1974). The hydrolysates were also analysed with an amino acid analyser to determine diaminopimelic acid (DAP) and fucosamine.

Electron microscopy. Growing cells were fixed by the addition of an equal volume of the fresh medium containing 8% (v/v) glutaraldehyde to the culture. The mixture was kept at room

Table 1. Bacterial strains used

Abbreviations: Thr⁻, threonine requirement; Met⁻, methionine requirement; Str^r, streptomycin resistance; Nal^r, nalidixic-acid resistance; TUA⁻, loss of TUA; TUP-Glu⁻, loss of poly(glutamic acid) chain in TUP.

Strain	Phenotype	Reference
C-125	Wild-type	Aono & Horikoshi (1983)
C-125-11	Thr ⁻ Str ^r TUA ⁻	Aono & Ohtani (1990)
C-125-90	Thr ⁻ Str ^r TUA ⁻ TUP-Glu ⁻	Aono & Ohtani (1990)
C-125-F19	Met ⁻ Str ^r Nal ^r TUP-Glu ⁻	Ito <i>et al.</i> (1994)

temperature for 1–2 h and then at 4 °C overnight. Samples were sedimented by centrifugation (1000 *g*, 15 min, 20 °C) and resuspended in 0.1 M sodium cacodylate buffer (pH 7.2) containing 4% (v/v) glutaraldehyde for several days. The fixed samples were washed with 0.1 M phosphate buffer (pH 7.2), post-fixed with OsO₄ at 4 °C, dehydrated and embedded in epoxy resin. Sections stained with uranyl acetate and costained with lead citrate were observed in a Philips 201C electron microscope.

RESULTS AND DISCUSSION

Extraction of the non-peptidoglycan components from the cell-wall-defective mutants

Cell walls from C-125 and C-125-11 were prepared from cells grown in 2 l alkaline medium to an OD₆₆₀ of 2. Cell walls from C-125-90 and C-125-F19 were prepared from cells grown in 2.5 l of the same medium to an OD₆₆₀ of 0.7–0.8. The cell-wall preparations contained the following amounts of uronic acids (μmol l⁻¹ OD₆₆₀⁻¹): C-

125, 53; C-125-11, 23; C-125-F19, 67; C-125-90, 55. The insoluble material remaining after repeated TCA extraction represents only 2–4% of the original uronic acids and consists mainly of peptidoglycans (Aono *et al.*, 1984). The TCA extracts contained little peptidoglycan.

After dialysis and anionic ion-exchange column chromatography, 90–95% of the solubilized uronic acid residues were recovered. High recoveries of uronic acids were also found for TCA extracts of cell-wall preparations from organisms grown in neutral medium (results not shown). These results indicate that the major source of uronic acid residues is the high-*M_r* polymers in the cell-wall preparations.

Characterization of cell-wall components of the cell-wall-defective mutants grown at pH 10

TUA and TUP found in the cell walls of wild-type strain C-125 can be separated from each other by DEAE-cellulose column chromatography (Aono, 1985). The

Fig. 1. DEAE-cellulose column chromatography of the non-dialysable TCA extract of cell walls. Cell walls were prepared from cells of C-125 (a), C-125-11 (b), C-125-F19 (c) or C-125-90 (d) grown in alkaline medium and extracted. The extracts were chromatographed as described in Methods. Fractions (3 ml) were collected and assayed for uronic acids (●), L-glutamic acid (○) and amino sugars (Δ). Each concentration is shown in mM.

Table 2. Characteristics of non-peptidoglycan acidic polymers in the bacterial cell walls

Cell-wall polymers extracted with TCA were purified by DEAE-cellulose chromatography. The M_r of each polymer was estimated by gel chromatography. The constituents of each polymer were identified by Avicel cellulose TLC and paper electrophoresis after hydrolysis. Abbreviations: PGU, polyglucuronic acid derived as a mutant-type TUP deprived of a polyglutamic acid chain; GlcU, glucuronic acid; GalU, galacturonic acid; L-Glu, L-glutamic acid.

Strain	Component	M_r	Constituents
C-125	TUA	44000	GlcU GalU FucN
	TUP	21000	GlcU L-Glu
C-125-11	TUP	21000	GlcU L-Glu
	PGU	7300	GlcU
C-125-90	TUP	21000	GlcU L-Glu
	PGU	7200	GlcU
C-125-F19	TUA	38000	GlcU GalU FucN
	TUP	21000	GlcU L-Glu

Fig. 2. Culture-pH dependence of the acidic constituents in cell walls. Strains C-125 (○), C-125-11 (●), C-125-F19 (△) and C-125-90 (▲) were grown at several pH values. The acidic and DAP levels in the cell walls were determined as described in Methods. The total amounts of acidic constituents (uronic acids and L-glutamic acid) are represented (mg peptidoglycan)⁻¹.

TUA, composed of galacturonic acid, glucuronic acid and fucosamine, eluted with 0.33 M NaCl. The TUP, which has glucuronic acid and glutamic acid as constituents, eluted with 0.45 M NaCl (Fig. 1a; Table 2).

In contrast, chromatography of the TCA extract of cell walls of C-125-11 yielded only one peak (Fig. 1b). This peak eluted with 0.45 M NaCl and consisted mainly of glucuronic acid and glutamic acid (Table 2), showing that the peak was derived from TUP. This proves that the cell walls of C-125-11 do not contain TUA and do not contain any equivalent polymer to replace the missing TUA. Previously, it has been assumed that the mutant C-125-11 lacks TUA because fucosamine was not detected in a hydrolysate of the cell walls of the strain (Aono & Ohtani, 1990).

TCA extracts from the mutant C-125-90 contained two polymers (Fig. 1d). One polymer eluted with 0.32 M NaCl and consisted of only glucuronic acid and a small amount of galactosamine residues (Table 2). This compound was PGU with an M_r of 7100 and corresponds to the PGU moiety of TUP, which was previously prepared by hydrazinolysis of TUP (Aono, 1989). The M_r was greater than the value of 4800 determined for the hydrazinolysis product. It is possible that the PGU moiety had been partially decomposed during the hydrazinolysis in the previous study. Another cell-wall polymer of C-125-90 (Fig. 1d) eluted with 0.45 M NaCl and is TUP. The chemical composition and M_r are similar to those of the TUP prepared from the wild-type strain (Table 2). The TUP levels varied considerably between different cultures of C-125-90. C-125-90 readily produces a revertant similar to C-125-11 at high pH. This revertant had TUP but not TUA or PGU (results not shown). It is believed that the TUP levels found here are from the revertant rather than from C-125-90. An alternative explanation is that C-125-90 is a mutant leaky in TUP synthesis. Recently we have cloned a DNA fragment from the wild-type C-125 that complements the defect in TUP synthesis in the mutants C-125-90 and C-125-F19 (results not shown). Future analysis of this DNA fragment may reveal characteristics of the mutation and the gene products involved in TUP synthesis.

DEAE-cellulose chromatography of the TCA extracts from C-125-F19 showed that this mutant contained TUA, TUP and PGU (Fig. 1c). The highly acidic fraction that eluted with 0.4 M NaCl consisted of a TUP identical to that extracted from C-125, as found in C-125-90. The polymers present in the low-acidic-component fraction that eluted with 0.3 M NaCl were further fractionated by gel chromatography on Toyopearl HW55S to yield two substances (results not shown). The higher M_r polymer was TUA of a chemical composition identical to that of wild-type TUA. The lower M_r polymer was PGU (a mutant-type TUP consisting only of PGU chains) identical with that extracted from C-125-90 cell walls (Table 2). C-125-F19 has been constructed by cell fusion of protoplasts from C-125-90 and the wild-type strain (Aono *et al.*, 1994; Ito *et al.*, 1994), and its cell-wall polymers reflect the parentage of the fusion product.

The results described above for the cell-wall polymers are for cells grown at pH 10. Similar results were obtained for cells grown at pH 7, but the amounts of acidic polymers were much reduced in this case (see later).

Fig. 3. Culture-pH dependence of acidic polymers in cell walls. Contents of TUA (broken line), TUP (solid line), and PGU (broken line and dot) in the cell walls of strains C-125 (○), C-125-11 (●), C-125-F19 (△) and C-125-90 (▲) were calculated from the chemical compositions of the cell-wall preparations (Table 3). The polymer levels are presented (mg peptidoglycan)⁻¹.

Culture-pH-dependent accumulation of the acidic constituents in the cell walls

The chemical composition of cell walls from the alkaliphilic *B. lentus* C-125 grown at pH 7 or 10 has been reported previously by Aono & Horikoshi (1983) and Aono (1985). Uronic acids are the major constituents of the acidic non-peptidoglycan polymers TUA, TUP and PGU. Fucosamine, L-glutamic acid and DAP are unique constituents of TUP, TUA and peptidoglycan, respectively. In the present study, the constituents of cell walls prepared from mutants defective in cell-wall components grown at six pH values were measured in order to evaluate the influence of culture pH on the acidic polymer concentrations (Figs 2 and 3; Table 3). The pH values were kept constant during the growth. The amount of peptidoglycan was calculated by multiplying the measured DAP content by 884 ($\mu\text{g } \mu\text{mol}^{-1}$). This factor has been experimentally determined on the basis of the chemical composition of peptidoglycan prepared from strain C-125 (Aono *et al.*, 1984). In the cell walls, fucosamine is *N*-acetylated (Aono, 1985). Galacturonic acid, glucuronic acid and glutamic acid residues occur as monovalent acidic residues in the polymers. Therefore, the anionic charge on cell walls can be approximately estimated from the total amount of acidic compounds.

The results listing the levels of L-glutamic acid, uronic acids and fucosamine relative to DAP in the cell walls are given in Table 3, together with the total anionic charge concentrations. The results show that the cell walls of the

Table 3. Culture-pH dependence of the non-peptidoglycan components of cell walls

The cell walls were prepared from bacteria grown at the constant pH indicated and assayed for constituents of TUA, TUP and peptidoglycan. The L-glutamic acid (L-Glu), uronic acids (UA) and fucosamine (FucN) contents relative to the DAP content are shown. ND, Not detected.

Strain	Culture pH	Amounts relative to DAP ($\mu\text{mol } \mu\text{mol}^{-1}$)			
		L-Glu	UA	FucN	Total anionic compounds
C-125	6.8	1.50	0.51	0.08	2.0
	7.0	2.10	0.69	0.12	2.8
	8.0	2.90	1.70	0.36	4.6
	9.0	3.70	1.90	0.49	5.6
	10.0	4.10	2.10	0.49	6.2
	10.5	4.40	2.40	0.46	6.8
C-125-11	6.8	3.40	0.74	ND	4.1
	7.0	3.40	0.79	ND	4.2
	8.0	4.10	0.90	ND	5.0
	9.0	4.30	0.91	ND	5.2
	10.0	4.50	1.00	ND	5.5
	10.5	4.60	1.10	ND	5.7
C-125-F19	6.8	0.47	1.20	0.19	1.7
	7.0	0.53	1.30	0.07	1.8
	8.0	0.50	2.20	0.19	2.7
	9.0	0.53	2.30	0.15	2.8
	10.0	0.80	2.90	0.23	3.7
C-125-90	6.8	0.46	1.10	ND	1.6
	7.0	0.51	1.30	ND	1.8
	8.0	0.65	1.80	ND	2.4
	9.0	0.92	1.80	ND	2.7
	10.5	1.75	2.10	ND	3.8

four alkaliphilic strains grown in high alkaline environments were highly anionic compared to the cell walls from the bacteria grown in neutral environments. In the case of C-125, L-glutamic acid was the predominant acidic compound and this increased almost threefold with respect to DAP when the culture pH increased from 6.8 to 10.5. The relative concentrations of uronic acids increased over fourfold. The total acidic constituents in the cell walls increased from 2.0 $\mu\text{mol (}\mu\text{mol DAP)}^{-1}$ in cells grown at pH 6.8 to 6.8 $\mu\text{mol (}\mu\text{mol DAP)}^{-1}$ in cells grown at pH 10.5.

Similarly, the amounts of acidic cell-wall compounds increased with culture pH for each of the mutants, although the amounts of anionic compounds in the three mutants increased less with increasing culture pH than was found for the wild-type. C-125-11 had highly anionic cell walls irrespective of the culture pH due to overproduction of TUP (Fig. 3; Table 3). This mutant had 4.1 $\mu\text{mol acidic compounds (mg peptidoglycan)}^{-1}$ even when grown at pH 6.8. The amount of acidic compounds

of strains C-125-F19 and C-125-90 was $1.6\text{--}1.7\text{ }\mu\text{mol (mg peptidoglycan)}^{-1}$ at pH 6.8 and $3.7\text{--}3.9\text{ }\mu\text{mol (mg peptidoglycan)}^{-1}$ at pH 10.

To assess the effects of culture pH on the amounts of TUA and TUP in the cell walls, the TUA and TUP levels were calculated by multiplying the fucosamine and L-glutamic acid contents by 539 and 168 ($\mu\text{g }\mu\text{mol}^{-1}$), respectively. These factors were determined from the chemical composition of the free-acid forms of TUA and TUP (Aono, 1985, 1989). PGU, which appeared only in the cell walls of C-125-90 and -F19, was composed mainly of glucuronic acid (Table 2). Therefore, the PGU content was estimated by multiplying the excess uronic-acid content by 176 ($\mu\text{g }\mu\text{mol}^{-1}$). This excess uronic acid was the residue not involved in formation of TUA or TUP. The excess uronic-acid residue content was calculated by subtraction of the uronic-acid content in TUA (twice that of the fucosamine content) and subtraction of the uronic-acid content of TUP (22% that of the L-glutamic acid content) from the total content of uronic acids in the cell walls.

The estimated TUA, TUP and PGU content per unit of peptidoglycan in the cell walls of the four strains and the changes in their concentration with culture pH are presented in Fig. 3. The acidic polymers probably exist as the sodium or potassium salts in cells growing at high pH, but the results in Fig. 3 were calculated as the free-acid forms for convenience.

In C-125 cell walls, the TUA concentration increased in a linear-pH-dependent manner between pH 6.8 and 9.0 and then remained almost constant at higher pH (Fig. 3). The concentration of TUA was only $50\text{ }\mu\text{g (mg peptidoglycan)}^{-1}$ at pH 6.8 but increased to $300\text{ }\mu\text{g (mg peptidoglycan)}^{-1}$ at pH 9.0. In contrast, the TUP concentration increased over the whole pH range tested with a near-linear increase from pH 8 to 10.5. At pH 6.8 and 10.5, the TUP concentration was 280 and $830\text{ }\mu\text{g (mg peptidoglycan)}^{-1}$, respectively. These results show that culture pH affects the synthesis of both TUA and TUP, and suggest that the synthesis of the two polymers is not linked.

For C-125-11, the acidic polymer in the cell walls is only TUP (Fig. 1b). At pH 6.8 and 10.5, the respective TUP concentrations were 640 and $870\text{ }\mu\text{g (mg peptidoglycan)}^{-1}$. At pH values below 8, the TUP concentration was far greater than that in the parent strain (C-125). TUP synthesis possibly increased in response to low concentration of TUA. An enhanced TUP content might complement biological defects brought about by the lack of TUA, although little is known about the biological functions of TUP and TUA at present.

The mutant C-125-90 prepared by mutating strain C-125-11 also did not contain TUA. Cultures of C-125-90 grown at high pH often contained C-125-11-type revertant cells. It was difficult to interpret results from cultures of this mutant because of the presence of wild-type TUP (a complex of PGU and polyglutamic acid), which probably arose from the revertants. The cell-wall preparation contained 90 and $330\text{ }\mu\text{g wild-type TUP (mg peptidoglycan)}^{-1}$ when the organism was grown at pH 6.8 or

10.0, respectively. These values were equivalent to 0.004 and $0.016\text{ }\mu\text{mol (mg peptidoglycan)}^{-1}$. The cell walls of C-125-90 contained PGU, a mutant type of TUP lacking the polyglutamic acid chain (Fig. 1d). The PGU content was $200\text{ }\mu\text{g (0.028 }\mu\text{mol) (mg peptidoglycan)}^{-1}$ at pH 6.8 and $350\text{ }\mu\text{g (0.048 }\mu\text{mol) (mg peptidoglycan)}^{-1}$ at pH 10.0, which compared with respective values of 0.030 and $0.041\text{ }\mu\text{mol TUP (mg peptidoglycan)}^{-1}$ found in the cell walls of C-125-11 grown under these culture conditions.

For strain C-125-F19, wild-type TUP was found in the cell-wall preparation (Fig. 1c) but the amount was less than that in C-125-90 at pH 9 or 10 (Fig. 3). This probably indicates that C-125-F19 was more stable than C-125-90 at alkaline pH. This stability might be due to restoration of TUA and to a consequent increase in negative charges in the cell walls of strain C-125-F19. At pH 10.0 the amount of TUA was $120\text{ }\mu\text{g (mg peptidoglycan)}^{-1}$, which is about a half of that of C-125 grown at the same pH. The cell-wall preparation contained 90 and $150\text{ }\mu\text{g (0.004 and }0.007\text{ }\mu\text{mol, respectively) of wild-type TUP (mg peptidoglycan)}^{-1}$ when C-125-F19 was grown at pH 6.8 and 10.0, respectively. Cell walls also contained 150 and $450\text{ }\mu\text{g PGU (mg peptidoglycan)}^{-1}$ at pH 6.8 and 10.5, respectively. This corresponds to 0.021 and $0.062\text{ }\mu\text{mol PGU (mg peptidoglycan)}^{-1}$, respectively.

In summary, synthesis of the acidic polymers TUA and TUP increased markedly when the facultative alkaliphilic strain C-125 was grown at alkaline pH. TUA and TUP give anionic charges to the cell walls, and changes in the content of these polymers will alter the negative-charge density in the cell-wall matrix (Fig. 2). We previously compared the chemical composition of cell walls prepared from a neutrophilic strain of *B. subtilis* and 10 alkaliphilic strains of *Bacillus* spp. (Aono & Horikoshi, 1983). Teichoic acid containing phosphor was a major acidic constituent in the cell wall of *B. subtilis*. The phosphor content was $1.8\text{ }\mu\text{mol (mg cell wall)}^{-1}$ and $3.6\text{ }\mu\text{mol (}\mu\text{mol DAP)}^{-1}$. Anionic charges expected in the cell wall of *B. subtilis* due to the phosphor can be supposed to be similar to those of *B. lentus* C-125 grown at pH 7–8 (Table 3). The cell wall of C-125 grown at pH 10.5 was enriched in about twice the amount of acidic compounds as that in *B. subtilis*.

pH-dependent growth of mutants defective in cell-wall components

A series of growth studies was carried out in liquid medium under pH control to determine the growth rates of the cell-wall-defective mutants and the wild-type. At alkaline pH, the three mutants grew with lower growth rates than the parent strain (Fig. 4). C-125 did not grow at pH 6.5 or 11.0 when cultured under pH control, but grew between pH 6.8 and 10.8 with optimum growth at pH 9. The specific growth rates were 0.77, 1.33 and 0.34 h^{-1} at pH 6.8, 9.0 and 10.8, respectively. None of the mutant strains grew below pH 6.5.

Mutant strain C-125-11 grew between pH 6.8 and 10.7 with optimum growth at pH 8. This mutant did not grow at pH 10.8. The growth rate at pH 10.0 and 10.5 was 85%

Fig. 4. Effect of culture pH on growth of alkaliphilic *B. lentus* and its cell-wall-defective derivatives. Strains C-125 (○), C-125-11 (●), C-125-F19 (△) and C-125-90 (▲) were grown at several constant pH values. Specific growth rates were calculated from generation times derived from increases in turbidity (OD_{600} was measured).

and 60 % of that of C-125, respectively. Mutants C-125-F19 and C-125-90 grew between pH 6.8 and 10.5, and both showed growth optima at pH 7, with growth rates 90 % that of C-125. These mutants did not grow at pH 10.7 and had growth rates only 20 % of that of C-125 at pH 10.5 (Fig. 4).

At neutral pH, the growth rates of mutants and the wild-type were similar despite the differences in cell-wall constituents. The growth rates of the mutants at pH 7 were almost equal to that of C-125. Above pH 8, the growth rates of all the mutants continually reduced as pH increased, and the growth rates remained in the order C-125-11 > C-125-F19 > C-125-90. At their pH optima, the growth rates of the mutants were very similar to that of wild-type C-125 growing at the same pH. It appears that the alkaliphilic nature of the mutant strains has been reduced by the changes to the cell-wall constituents. However, these changes were not sufficient for the mutants to grow below the lower-pH limit for growth of C-125 or to grow more rapidly at neutral pH than C-125. Interestingly, the mutant with the most similar growth rates to C-125 is C-125-11, and this mutant has completely lost the TUA component of its cell walls. From the growth rate curves in Fig. 4, it can be seen that C-125-11 resembles C-125, whereas C-125-F19 resembles C-125-90. This resemblance reflects the similarities in the concentrations of anionic compounds in the cell walls of the four strains (Fig. 2; Table 3) and leads us to conclude again that TUA and TUP function on the cell surface of alkaliphilic bacteria as an obstacle to high concentrations of OH^- .

Ultrastructure of cell walls of alkaliphilic *B. lentus* C-125 and its mutants

The cell-wall features of the four strains in the exponential phase of growth were monitored by electron microscopy. *B. lentus* C-125 is Gram-positive and has a relatively thick cell-wall layer (Fig. 5a). The thickness and other morphological features of the cell walls varied with culture pH, although this phenomenon was observed on the micrographs showing dehydrated walls but not hydrated walls surrounding the growing cells. The dehydrated cell walls are likely to be thinner than the *in vivo* hydrated cell walls.

The cell walls of the organism grown at pH 10 (A-wall) were thicker than those of the organism grown at pH 7.2 (N-wall), as shown in Table 4. The A-wall was 22 nm in thickness, which was similar to that of *B. subtilis* cell walls (Mera *et al.*, 1992). Triple layers consisting of electron-dense, electron-opaque and electron-dense lamellae were well developed in the A-wall (Fig. 5b). This multiple lamellae structure was poorly developed in the N-wall and the electron-opaque layer between the two electron-dense layers was evident in only a few of the cells grown at pH 7 (Fig. 5c). The N-wall of most C-125 cells appeared as an electron-dense monolayer of 18 nm thickness. All the multiple layers disappeared upon digestion of the peptidoglycan with egg-white lysozyme (Aono *et al.*, 1994) but were not affected by trypsin (results not shown).

For mutant C-125-11, triple layers were also present in the A-walls (19 nm thickness) as shown in Fig. 5(d) but were less evident in the N-walls (13 nm thickness) of the mutant (results not shown). The middle layer found as an electron-opaque layer in C-125 cells and mutant C-125-11 grown at alkaline pH might be an area of peptidoglycan enriched in TUP because these cells were enriched in TUP (Fig. 3).

Triple layers were not found in A-walls (18 nm thickness; Fig. 5e) and N-walls (16 nm thickness) of C-125-F19 or in A-walls (17 nm thickness; Fig. 5f) or N-walls (14 nm thickness) of C-125-90. The cell walls of mutants C-125-F19 and C-125-90 consisted of a dense monolayer and this appeared to be little affected by changes in culture pH. The monolayers of these cell walls seemed to lack the electron-dense outermost lamella and the electron-opaque middle lamella found in the A-walls of strains C-125 and C-125-11 (Figs 5b, d). All four strains possessed relatively thick cell walls when they grew at alkaline pH.

Density of fixed negative charges in the cell-wall matrix

We previously described the culture-pH-dependent alteration of the chemical composition of cell walls of alkaliphilic strains of *Bacillus* spp. (Aono & Horikoshi, 1983; Aono, 1985). At the time, we were unable to interpret this alteration in terms of the negative-charge density provided by TUA and TUP because of the lack of information on the morphology of the cell walls. We supposed that the alteration might be one of the physio-

Fig. 5. Electron micrographs of cell walls of alkaliphilic *B. lentus* C-125 and its cell-wall-defective derivatives. Cells of C-125 (a-c), C-125-11 (d), C-125-F19 (e) and C-125-90 (f) were grown at pH 7 (c) or 10 (a, b, d-f). The fixation and staining procedure for the cells in the exponential phase of growth is given in Methods. Bars, 1.0 μm (a) or 0.1 μm (b-f).

Table 4. Cell-wall thickness of the derivatives

Electron micrographs of the cells grown at pH 7 or 10 were taken at magnifications of $\times 103\,600$ – $221\,900$. Cell-wall thicknesses were measured by using several micrographs. A mean value ($\bar{x}$) and standard deviation (SD) were calculated for 20–38 measurements of the cell-wall thickness.

Strain	Culture pH	$\bar{x} \pm \text{SD}$ (nm)
C-125	10	21.5 $\pm$ 2.2
	7	18.0 $\pm$ 3.1
C-125-11	10	18.9 $\pm$ 1.4
	7	12.9 $\pm$ 1.9
C-125-F19	10	18.4 $\pm$ 2.4
	7	15.8 $\pm$ 3.0
C-125-90	10	17.2 $\pm$ 1.9
	7	13.5 $\pm$ 1.6

logical adaptations of the organisms to alkaline environments. In the present study there is information available to calculate charge densities for the cell walls of the wild-type and its mutants, although the observed cell walls were likely to be shrunk due to the dehydration.

The cell morphology of strain C-125 depends on the culture pH. The unit cell is a rod of approximately $0.4 \times 1.3 \mu\text{m}$ at pH 10, whereas the cell is a short rod of approximately $0.6 \times 1.2 \mu\text{m}$ at pH 7. These cells have walls of 21.5 or 18.0 nm in thickness (Table 4). If the cell is assumed to have the shape of a simple cylinder, a volume can be calculated for the cell walls (the space external to the cytoplasmic membrane) in each type of cell. We have calculated that the volume of a cell-wall region of C-125 grown at pH 7 is about $4.9 \times 10^{-2} \mu\text{m}^3$ and the volume of a cell grown at pH 10 is about $3.8 \times 10^{-2} \mu\text{m}^3$.

A cell-wall preparation (2.22 g dry wt) from 2.2×10^{13} cells of strain C-125 grown at pH 7 contained 0.89 mmol uronic acids and 2.5 mmol L-glutamic acid. A cell-wall preparation (1.23 g dry wt) from 1.2×10^{13} cells grown at pH 10 contained 1.3 mmol uronic acids and 2.6 mmol L-glutamic acid. These results indicated that sum of the two acidic compounds was 1.5×10^{-16} mol per cell grown at pH 7 and 3.2×10^{-16} mol per cell grown at pH 10. If the acidic residues are assumed to be distributed homogeneously in the cell-wall layers, the fixed negative charge concentrations in the dehydrated cell walls can be calculated as 3.1×10^{-15} equivalents per μm^3 of cell wall region at pH 7 and 8.5×10^{-15} equivalents per μm^3 of cell wall at pH 10. These estimates including several assumptions might be higher than *in vivo* negative charge densities. However, the data are indicative of large differences in the negative charges on cell walls from the two preparations. It appears that the cell walls of C-125 grown in an alkaline environment (about 20% thicker than the cell walls at pH 7) have about three times the negative charge density of that in the cell walls of C-125

grown in a neutral environment. These differences are likely to have a major influence on the flux of anions and cations through the cell-wall matrix. In particular, the high negative charge density could contribute to lower pH in the innermost region of the cell walls by reserving hydrogen ions generated by respiration and released from the neighbouring cytoplasmic membrane (Koch, 1986; Kemper *et al.*, 1993).

ACKNOWLEDGEMENTS

This work was partially supported by a grant for the 'Biodesign Research Program' from RIKEN to R. Aono. The authors thank D. H. Hopcroft and R. J. Bennett of the Grasslands Research Centre for technical assistance with electron microscopy.

REFERENCES

- Aono, R. (1985). Isolation and partial characterization of structural components of the walls of alkaliphilic *Bacillus* strain C-125. *J Gen Microbiol* **131**, 105–111.
- Aono, R. (1987). Characterization of structural component of cell walls of alkaliphilic strain of *Bacillus* sp. C-125. *Biochem J* **245**, 467–472.
- Aono, R. (1989). Characterization of cell wall components of the alkaliphilic *Bacillus* strain C-125: identification of a polymer composed of polyglutamate and polyglucuronate. *J Gen Microbiol* **135**, 265–271.
- Aono, R. (1995). Assignment of facultatively alkaliphilic *Bacillus* sp. C-125 to *Bacillus lentus* group 3. *Int J Syst Bacteriol* **45**, 582–585.
- Aono, R. & Horikoshi, K. (1983). Chemical composition of cell walls of alkaliphilic strains of *Bacillus*. *J Gen Microbiol* **129**, 1083–1087.
- Aono, R. & Ohtani, M. (1990). Loss of alkaliphily in cell-wall-component-defective mutants derived from alkaliphilic *Bacillus* C-125. *Biochem J* **266**, 933–936.
- Aono, R. & Sanada, T. (1994). Hyper-autolysis of the facultative alkaliphile *Bacillus* sp. C-125 cells grown at neutral pH: culture-pH dependent cross-linking of the peptide moieties of the peptidoglycan. *Biosci Biotechnol Biochem* **58**, 2015–2019.
- Aono, R. & Uramoto, M. (1986). Presence of fucosamine in teichuronic acid of the alkaliphilic *Bacillus* strain C-125. *Biochem J* **233**, 291–294.
- Aono, R., Horikoshi, K. & Goto, S. (1984). Composition of the peptidoglycan of alkaliphilic *Bacillus* spp. *J Bacteriol* **157**, 688–689.
- Aono, R., Ito, M. & Horikoshi, K. (1992). Instability of the protoplast membrane of facultative alkaliphilic *Bacillus* sp. C-125 at alkaline pH values below the pH optimum for growth. *Biochem J* **285**, 99–103.
- Aono, R., Ito, M. & Horikoshi, K. (1993). Regeneration of protoplasts prepared from alkaliphilic strains of *Bacillus* spp. *Biosci Biotechnol Biochem* **57**, 1597–1598.
- Aono, R., Ito, M., Joblin, K. & Horikoshi, K. (1994). Genetic recombination after cell fusion of protoplasts from the facultative alkaliphile *Bacillus* sp. C-125. *Microbiology* **140**, 3085–3090.
- Beutler, H. O. & Michal, G. (1974). Glutamate, determination with glutamate dehydrogenase, diaphorase and tetrazolium salts. In *Methods of Enzymatic Analysis*, 2nd edn, vol. 4, pp. 1708–1713. Edited by H. U. Bergmeyer. Weinheim: Verlag Chemie.
- Davidson, E. A. (1966). Analysis of sugars found in mucopolysaccharides. *Methods Enzymol* **8**, 52–60.
- Ito, M., Tabata, K. & Aono, R. (1994). Construction of a new

teichuronopeptide-defective derivative from alkaphilic *Bacillus* sp. C-125 by cell fusion. *Biosci Biotechnol Biochem* **58**, 2275–2277.

Kemper, M. A., Urrutia, M. M., Beveridge, T. J., Koch, A. L. & Doyle, R. J. (1993). Proton motive force may regulate cell wall-associated enzymes of *Bacillus subtilis*. *J Bacteriol* **175**, 5690–5696.

Kitada, M., Hashimoto, M., Kudo, T. & Horikoshi, K. (1994). Properties of two different Na⁺/H⁺ antiport systems in alkaliphilic *Bacillus* sp. strain C-125. *J Bacteriol* **176**, 6464–6469.

Koch, A. L. (1986). The pH in the neighborhood of membranes generating a protonmotive force. *J Theor Biol* **120**, 73–84.

Mera, M. U., Kemper, M., Doyle, R. & Beveridge, T. J. (1992). The membrane-induced proton motive force influences the metal

binding ability of *Bacillus subtilis* cell wall. *Appl Environ Microbiol* **58**, 3837–3844.

Stepka, W. (1957). Identification of amino acids by paper chromatography. *Methods Enzymol* **3**, 504–528.

Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). Detection of sugars on paper chromatograms. *Nature* **166**, 444–445.

Wheat, R. W. (1966). Analysis of hexosamines in bacterial polysaccharides by chromatographic procedures. *Methods Enzymol* **8**, 60–78.

Received 3 March 1995; revised 14 July 1995; accepted 19 July 1995.