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Loss of alkalophily in cell-wall-component-defective mutants derived from alkalophilic *Bacillus* C-125

Isolation and partial characterization of the mutants

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The cells of alkalophilic *Bacillus* sp. C-125 are shaped by peptidoglycan and enclosed by two acidic polymers (teichuronic acid and teichuronopeptide), which bind to the peptidoglycan. Three kinds of mutant strains defective in these acidic polymers were isolated from the strain C-125. These mutants grow poorly at alkaline pH to extents related to the degree of defect in the polymers, suggesting that these acidic polymers are essential for growth in an alkaline environment. These polymers may diminish penetration of hydroxide ions.

INTRODUCTION

Each individual organism has a particular pH range at which it can grow or survive. This pH range is specified for each organism, but the mechanisms which prescribe the range are unclear. Alkalophilic micro-organisms grow well at alkaline pH, as indicated by their definition. The intracellular pH of alkalophilic organisms grown in alkaline media is kept roughly neutral (Guffanti *et al.*, 1978). There is a pH difference between the inside and outside of the cells, and this pH difference must be due to surface components or functions. An Na^+/H^+ antiporter on the cell membrane seems to take part in maintaining intracellular pH (Krulwich *et al.*, 1982). Other mechanisms may be needed on the cell surface, for example, an obstacle against high concentrations of hydroxide ion (OH^-).

Cell walls of alkalophilic *Bacillus* sp. C-125 are composed of peptidoglycan, teichuronic acid (TUA) and teichuronopeptide (TUP). The peptidoglycan is an A1γ type identical with that of neutrophilic *B. subtilis* in its chemical structure (Aono *et al.*, 1984). TUA is composed of galacturonic acid, glucuronic acid and *N*-acetyl-D-fucosamine in a molar ratio of 1:1:1 (Aono & Uramoto, 1986). TUP is a complex composed of polyglucuronic acid and poly(γ-L-glutamic acid) in an approximate molar ratio of 1:4–5 (Aono, 1985, 1987, 1989). Amounts of these acidic polymers are enhanced in the cell walls when the strain C-125 is grown at alkaline pH. Previously, one of us (Aono, 1985) pointed out the biological interest of the acidic polymers. The highly negative charges on the acidic non-peptidoglycan components enclosing the cell-wall peptidoglycan of the organism could form an anionic environment around the cells and give the cell surface its ability to adsorb Na^+ and hydronium ions and repulse OH^-, and as a result could enable the cells to grow in an alkaline environment. The anionic environment around the cells could function as an obstacle against OH^-.

The present paper describes the isolation and properties of mutants defective in the acidic polymers found in the cell walls; the results obtained support the hypothesis outlined above.

MATERIALS AND METHODS

Bacterial strains

All strains used are listed in Table 1. All mutant strains are derivatives of alkalophilic *Bacillus* sp. C-125 (deposited as FERM 7344 at The Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba, Japan). These mutant strains were obtained in the present study as described below.

Media and growth conditions

Alkaline complex medium contained, per litre of deionized water: K_2HPO_4, 13.7 g; KH_2PO_4, 5.9 g; citric acid, 0.34 g; $MgSO_4,7H_2O$, 0.05 g; glucose, 5 g; peptone, 5 g; yeast extract, 5 g; and Na_2CO_3, 10.6 g. Neutral complex medium was the same medium containing 11.7 g of NaCl instead of Na_2CO_3 (Aono, 1985). Alkaline synthetic medium previously used (Aono & Horikoshi, 1983) was modified as follows: K_2HPO_4, 13.7 g; KH_2PO_4, 5.9 g; citric acid, 0.34 g; $MgSO_4,7H_2O$, 0.05 g; glucose, 5 g; $(NH_4)_2SO_4$, 1 g; KNO_3, 1 g (per litre of deionized water). The pH of this synthetic medium was about 10. The same medium containing 11.7 g of NaCl instead of Na_2CO_3 was used as neutral synthetic medium at pH 7.2.

Isolation of mutants

(1) Marker mutants. Cells of *Bacillus* sp. C-125 growing in the alkaline complex medium at 37 °C were incubated in the following solution at 30 °C for 1 h; K_2HPO_4, 13.7 g; KH_2PO_4, 5.9 g; citric acid, 0.34 g; $MgSO_4,7H_2O$, 0.05 g; Na_2CO_3, 10.6 g; the mutagen ethyl methanesulphonate, 10 ml (per litre of deionized water). After

Abbreviations used: TUA, teichuronic acid; TUP, teichuronopeptide

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Table 1. Bacterial strains

The following abbreviations are used; Thr^-, requirement for threonine; Str^r, streptomycin-resistance; TUA^-, loss of TUA; $TUP\text{-}Glu^-$, loss of the poly(glutamic acid) region in TUP.

Strain	Phenotype
C-125	Wild-type
C-125-001	Thr^-
C-125-002	Thr^- Str^r
C-125-11	Thr^- Str^r TUA^-
C-125-23	Thr^- Str^r TUA^-
C-125-90	Thr^- Str^r TUA^- $TUP\text{-}Glu^-$

mutagenesis, auxotrophic mutants were concentrated by the penicillin-screening method and then selected by the replica method using the alkaline synthetic medium.

A spontaneous streptomycin-resistant mutant was isolated from one of the auxotrophic mutants on the neutral complex medium containing streptomycin (1 mg/l).

(2) TUA-defective mutant. A double marker mutant was treated with ethyl methanesulphonate as described above. Several strains which grew on the medium and formed colonies different in appearance from the parent strain were thoroughly selected and grown in 1 ml of the neutral medium. The cells were extracted with 5% (w/v) trichloroacetic acid at 80 °C for 20 min. Uronic acids in the extract were determined. Strains in which uronic acid contents seemed low were again grown in 100 ml of the neutral medium. Cell-wall fractions were prepared as described below. Components of the cell wall were analysed by determinations of uronic acids, L-glutamic acid, amino sugars and L-alanine and t.l.c.

(3) TUP-defective mutants. Cells of TUA-defective mutant were subjected to mutagenesis and suspended in cold 10 mM-phosphate buffer, pH 7.2, containing 0.2 mM-$MgSO_4$. The suspension was passed through a small column (0.5 cm × 5 cm) of Dowex 1-X4 (200–400 mesh; acetate form). Cells in the effluent were grown in the neutral complex medium containing streptomycin. After enrichment of low-anionic-surface cells were carried out more 4 times, the mutated cells were grown on the neutral complex medium. Cell-wall components of strains selected at random were analysed as described above.

Analyses

(1) Cell walls were prepared from the organisms grown aerobically at 30 °C by inactivation of autolytic enzymes with SDS at 60 °C, disruption with a sonic oscillator and tryptic digestion, as described previously (Aono & Horikoshi, 1983).

(2) Non-peptidoglycan acidic polymers were extracted with 5% (w/v) trichloroacetic acid, dialysed against water and fractionated on a DEAE-cellulose column, as described previously (Aono, 1985).

(3) Uronic acid was determined by the carbazole/H_2SO_4 method and expressed as glucuronic acid (Aono & Horikoshi, 1983).

(4) Fucosamine and diaminopimelic acid were analysed with an amino acid analyser after samples had been hydrolysed in 4 M-HCl at 105 °C for 15 h. L-Glutamic acid was determined with glutamate dehydrogenase. Amino sugars were also determined by the Elson–Morgan reaction as glucosamine (Aono, 1985).

(5) An ascending chromatogram was run on an Avicel cellulose thin-layer plate at room temperature, with ethyl acetate/pyridine/water/acetic acid (5:5:3:1, by vol.) as solvent. The components were located with a ninhydrin spray.

RESULTS AND DISCUSSION

Isolation of double-marker mutant

First, we established conditions under which alkalophilic *Bacillus* C-125 could be effectively mutated and devised a synthetic medium for the alkalophilic strain in order to obtain auxotrophic mutants. The strain was treated with ethyl methanesulphonate at alkaline pH. Several auxotrophic mutants could be enriched with potassium benzylpenicillin (3 mg/ml) and isolated by using the synthetic medium. A threonine-requiring mutant (C-125-001) was used to isolate a spontaneous streptomycin-resistant mutant (C-125-002).

Isolation of a TUA-defective mutant

It could be expected that colonies of cell-wall-defective mutants would be visibly different from those of the wild-type strain. Mutated strain C-125-002 was grown on the neutral complex medium without any enrichment of cell-wall-defective mutants. About 1% of the colonies showed different appearance on the medium. Among the apparently different colonies, 1000 strains were grown in the neutral medium. Cell-wall components were extracted with hot trichloroacetic acid directly from cells and were analysed. Among these 1000 strains, contents of uronic acids of 91 strains seemed low on the basis of their growth mass measured as A_{550}. Cell-wall fractions were prepared from the 91 strains and their chemical compositions were examined.

Only one mutant with defects in cell-wall components (mutant C-125-11) was isolated. Phenotypic mutation in the chemical composition of the cell wall was not clearly found in the other 90 strains. Therefore this mutant was obtained at a frequency of about 10^{-5}. Fucosamine was not found in its cell-wall fraction, indicating a loss of, or alteration in, TUA (Table 2). No TUA was found in the trichloroacetic acid extract of mutant C-125-11 (Table 3). Peptidoglycan of C-125-11 was the same as that of C-125 in its chemical composition (results not shown). These results indicated that this stain had completely lost TUA.

The mutant C-125-11 is probably defective in enzyme(s) involved in the synthesis of either a component sugar of the TUA or linkage between two components. On the other hand, the molar ratio of L-glutamic acid to diaminopimelic acid was enhanced in comparison with the parent strain (Table 2). The TUP content was increased in its cell wall, probably owing to secondary spontaneous mutation to compensate for the decrease of negative charges consequent on the loss of the TUA.

Isolation of TUP-defective mutants

The C-125-11 strain was subsequently treated with the mutagen in order to decrease the TUP content in the cell walls. Cells maintaining a high anionic charge on their surfaces could be selectively removed by adsorption

Table 2. Components of cell walls from alkalophilic *Bacillus* C-125 and its mutant strains

Each strain was grown at 30 °C in alkaline complex medium. Cell walls were prepared from cells in the early stationary phase of growth as described in the Materials and methods section. Diaminopimelic acid in cell-wall preparations was determined with an automatic amino acid analyser after hydrolysis in 4 M-HCl at 100 °C for 15 h to estimate the amount of peptidoglycan. The fucosamine content was determined with the amino acid analyser after the hydrolysis to estimate TUA. L-Glutamic acid was determined with L-glutamate dehydrogenase after the hydrolysis to estimate TUP. Uronic acids were determined by the carbazole/H_2SO_4 method. Values refer to dry weight of the cell-wall preparation and are given in nmol/mg. Molar ratios shown in parentheses were calculated by taking diaminopimelic acid as 1.0. N.D., not determined

Component / Strain...	Composition (nmol/mg) C-125	C-125-11	C-125-23	C-125-90
L-Glutamic acid	1520 (4.13)	2310 (5.98)	1960 (4.02)	487 (0.88)
Fucosamine	201 (0.55)	N.D.	N.D.	N.D.
Uronic acids	996 (2.71)	525 (1.36)	624 (1.28)	1520 (2.75)
Diaminopimelic acid	367 (1.0)	386 (1.0)	489 (1.0)	553 (1.0)

Table 3. Non-peptidoglycan acidic polymers found in the cell-wall-defective mutants

The acidic polymers bound to peptidoglycan were extracted with trichloroacetic acid. The non-diffusible fraction of the extract was fractionated by DEAE-cellulose column chromatography. The concentration of NaCl at which the peak of each compound was eluted is represented. Approximate molar proportions in the peak elution are represented by taking uronic acid as 1.0. Amino sugars determined by the Elson–Morgan reaction are represented as glucosamine. Abbreviations: UA, uronic acids; AS, amino sugars; Glu, L-glutamic acid; PG, polyglucuronate.

Strain	Compound	[NaCl] (M)	Approximate molar proportions (UA/AS/Glu)
C-125	TUA	0.32	1:0.4:0.001
	TUP	0.44	1:0.04:4
C-125-11	TUP	0.46	1:0.1:4
C-125-23	TUP	0.46	1:0.1:4
C-125-90	PG	0.33	1:0.04:0.1
	TUP	0.45	1:0.1:4

with Dowex-1 resin. Cells with lower anionic charge were enriched in the effluent from the column and grown in the neutral medium. The enrichment was carried out five times. After the fifth enrichment, 135 strains were obtained on the neutral medium. These strains were grown in the neutral medium. Cell-wall components were extracted with trichloroacetic acid. L-Glutamic acid in the extract was determined after the extract had been washed with diethyl ether. The L-glutamic acid content in the extracts from 20 strains seemed low on the basis of their growth mass, although the L-glutamic acid could also be derived from protein. Cell-wall fractions were prepared from the 20 strains and chemically analysed.

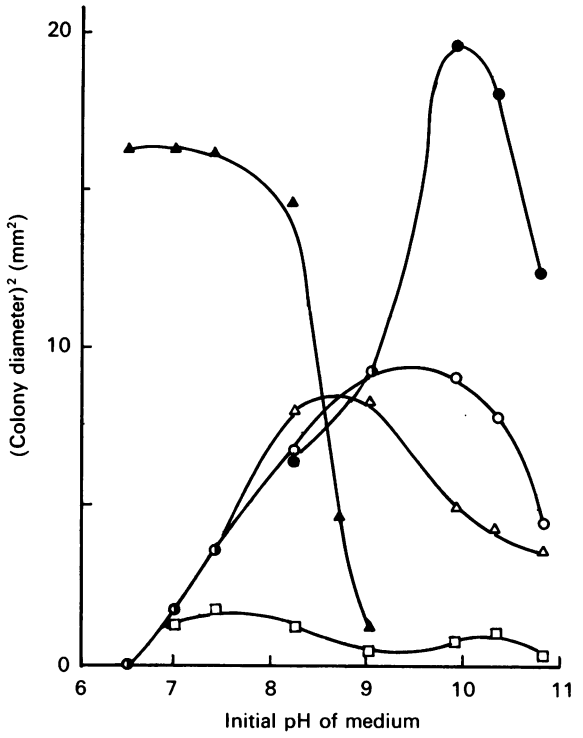

Fig. 1. Effect of culture pH on growth of alkalophilic *Bacillus* sp. C-1235 and its derivatives

Each strain was grown in the neutral complex medium at 37 °C overnight. The culture was diluted with 0.1 M-phosphate buffer, pH 7.2, and plated on a complex medium containing 5 g of peptone, 5 g of yeast extract, 5 g of glucose, 0.34 g of citric acid and 0.05 g of $MgSO_4,7H_2O$ as a basal medium. The medium was supplemented with 0.2 M-NaCl and 0.1 M-K_2HPO and adjusted with phosphoric acid to pH 6.5–7.5 or with Na_2CO_3 to pH 8. It was supplemented with 0.1 M-$NaHCO_3$, 0.05 M-Na_2CO_3 and 0.1 M-K_2HPO_4 for pH 9; 0.2 M-KCl and 0.1 M-Na_2CO_3 for pH 10; 0.2 M-KCl, 0.15 M-Na_2CO_3 and 0.05 M-NaOH for pH 10.5; 0.2 M-KCl, 0.05 M-Na_2CO_3 and 0.15 M-NaOH for pH 11. The actual pH value of the surface of each medium was determined with the flat type of glass electrode. The diameters of 20 colonies on each medium were measured after incubation at 37 °C for 24 h. The Figure shows a plot of the square of the average diameter of the colonies of the wild-type strain (●), C-125-11 (○), -23 (△) and -90 (□) against pH. *B. subtilis* GSY1026 (▲) was used as reference.

Two mutants with lowered molar ratios of L-glutamic acid to diaminopimelic acid were isolated. This ratio was not lowered in the other 18 strains. Therefore this type of mutant could be obtained at a frequency of 1.5×10^{-2} by enrichment. Peptidoglycans were identical with those of the wild-type strain (results not shown). Contents of diaminopimelic acid were enhanced (Table 2). The ratio of L-glutamic acid to diaminopimelic acid in the cell wall from C-125-23 was similar to that of the wild-type strain. The TUP molecules prepared from the parent strain C-125 and the mutant strains C-125-11 and C-125-23 were

identical with respect to their negative charges and chemical compositions (Table 3). It was concluded that C-125-23 had TUP decreased to the same amount as in the wild-type strain.

In the other strain (C-125-90), the L-glutamic acid content decreased remarkably and the uronic acid content was increased in its cell-wall fraction, indicating an alteration in TUP structure (Table 2). A novel anionic polymer composed mainly of uronic acid was found in the trichloroacetic acid extract of C-125-90 (Table 3). This novel polymer was a polyglucuronic acid element of the TUP almost totally deprived of the poly(glutamic acid) region, although a little amino sugar and L-glutamic acid remained. A smaller amount of 'wild-type' TUP was recovered together with the novel polymer (Table 3). The strain C-125-90 was prone to revert, because it grew poorly, as described below. The wild-type TUP was probably produced by revertant cells. The mutant C-125-90 did not require L-glutamic acid. Therefore the mutant is probably defective in enzyme(s) involved in the elongation of the poly(glutamic acid).

Growth of mutant strains at various pH values

Growth of these parent and mutant strains was examined on complex media at various pH values (Fig. 1). The wild-type strain C-125 showed a growth optimum at pH 10 and did not grow at pH 6.5. None of the mutant strains could grow below pH 6.5. Growth optima of the mutant strains C-125-11, -23 and -90 were at pH 9, 8.5 and 7.5 respectively. These optima were lowered with increasing defectiveness of the acidic polymers. The growth of the mutant strains at their optima was similar to that of the wild-type strain at its optimum. These strains grew very poorly in a higher-pH region above their respective growth optima pH. They grew similarly to the wild-type strain in the lower-pH region. Growth of the mutants was not enhanced at the lower pH region, as found in neutrophilic *B. subtilis*. It is therefore concluded that these mutant strains have lost their alkalophily but have not become neutrophilic.

The results described in here indicate that the acidic polymers in the cell walls of alkalophilic *Bacillus* sp. C-125 contribute to the ability of the strain to grow at alkaline pH. We hypothesize that TUA and TUP function on the cell surface of the organism as obstacles against high concentrations of OH^-, as previously suggested (Aono, 1985).

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