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Identification of facultatively alkaliphilic *Bacillus* sp. strain YN-2000 and its fatty acid composition and cell-surface aspects depending on culture pH

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Abstract Facultatively alkaliphilic *Bacillus* sp. strain YN-2000 was isolated from an indigo ball. Although the strain has been extensively investigated as a representative strain of alkaliphilic bacillus, its taxonomic position is not yet known. Morphological, biochemical, and physiological characteristics and chemotaxonomic properties indicated that the strain was closely related to *Bacillus cohnii*; this was confirmed by the high homology of the 16*S* rRNA sequence and the construction of a phylogenetic tree on the basis of the 16*S* rRNA sequence and DNA–DNA relatedness data. Strain YN-2000 contained a larger amount of unsaturated fatty acids compared with *Bacillus subtilis* and the obligate alkaliphile, *Bacillus alcalophilus*, regardless of its culture pH. When the cells were grown at pH 10, the unsaturated fatty acid content and anteiso-/iso-branched fatty acid ratio became lower than those at pH 7. This result suggests that membrane fluidity decreases when the cells are grown at pH 10 compared to those of pH 7. In the cells of strain YN-2000 grown at pH 10, the cell-surface aspect was rougher, the cell shape was longer, and the cell-surface layer was thicker compared with those of the cells grown at pH 7. The cell-surface structural change might be related to adaptation to an alkaline environment.

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Key words *Bacillus cohnii* · Alkaliphilic · 16*S* rRNA analysis · DNA–DNA relatedness · Phylogenetic tree

Introduction

A large number of alkaliphiles have been isolated from a variety of environments for industrial applications and studies of physiology for adaptation to an alkaline environment. However, most of the strains have not been identified up to the species level since Vedder (1934) isolated *Bacillus alcalophilus*. Recently, more than ten novel alkaliphilic *Bacillus* species were proposed on the basis of phenotypic characteristics, DNA–DNA relatedness data, and phylogenetic analysis based on the 16*S* rRNA sequence (Agnew et al. 1995; Fritze 1996; Garabito et al. 1997; Nielsen et al. 1995; Spanka and Fritze 1993; Switzer Blum 1998; Yumoto et al. 1998b). Several alkaliphilic *Bacillus* strains have been studied as representative strains for understanding the mechanisms of alkaliphily. Among the strains, *Bacillus firmus* OF4 (Guffanti et al. 1986) was extensively investigated in terms of bioenergetics (Krulwich et al. 1996) and cytoplasmic pH regulation (Krulwich et al. 1997). Recently, this strain was reclassified as *Bacillus pseudofirmus* (Takami and Krulwich 2000). The cell wall components (Aono et al. 1995) and ion-transport systems (Kitada et al. 1994) of *Bacillus lentus* C-125 (Aono 1995) have been studied extensively. Recently, this strain was reclassified as *Bacillus halodurans* (Takami and Horikoshi 1999). Although the respiratory systems (Higashibata et al. 1998; Orii et al. 1991; Qureshi et al. 1990, 1996; Yumoto et al. 1991, 1993), catalase (Yumoto et al. 1990), solute transport system (Koyama and Nosoh 1985; Koyama et al. 1987; Wakabayashi et al. 1988), flagella rotatory system (Sugiyama et al. 1986), and polyamines (Hamasaki et al. 1993) have been studied in *Bacillus* sp. strain YN-2000, the taxonomic position at the species level remains unknown for this strain. Identification of the taxonomic position of strain YN-2000 is quite important for carrying out comparative studies with other alkaliphilic *Bacillus* strains such as those already described.

In the present study, we attempted to identify strain YN-2000 and analyzed its fatty acid composition and observed its cell-surface aspects depending on culture pH.

Materials and methods

Bacterial strains and cultivation

The strain we examined was *Bacillus* sp. strain YN-2000, which was obtained from an indigo ball. The strain was isolated and identified as *Bacillus* sp. by Ohta et al. (1975). In addition to strain YN-2000, *Bacillus cohnii* DSM 6307^T was used as a reference for phenotypic characteristics, DNA–DNA relatedness, and fatty acid analysis. In addition, *Bacillus alcalophilus* JCM 5262^T and *Bacillus subtilis* IAM 1026 were used as reference strains for fatty acid composition. The alkaliphilic strains were cultivated aerobically until the late logarithmic growth phase at 30°C in a PYA (peptone-yeast extract-alkaline) medium (pH 10) (Yumoto et al. 1998b), unless otherwise stated. Growth at pH 7 for neutrophile and facultatively alkaliphilic strains was performed in a PYA medium containing 100 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7).

Phenotypic and chemotaxonomic characterization

Phenotypic characterization and analyses of cellular fatty acids and isoprenoid quinones were performed as previously described (Yumoto et al. 1998b), unless otherwise stated. Aminopeptidase activity (test strips; Merck, Darmstadt, Germany), pullulanase tests, and 4-methylumbelliferone glucuronidase (MUG) were performed by Cerny (1978), Morgan et al. (1979), and Feng and Hartmann (1982), respectively.

Identification of *meso*-diaminopimelic acid in the cell wall was performed by thin-layer chromatography (TLC) (art. 5552 DC-Alufoline Cellulose; Merck), as described by Yamada and Komagata (1970). Bacterial DNA was prepared according to the method of Marmur (1961). The DNA obtained was digested with nuclease P1 (Yamasa Shoyu, Choshi, Japan) and the resulting nucleotides were separated by high-performance liquid chromatography (HPLC) (Tamaoka and Komagata 1984).

16S rRNA sequencing

The 16*S* rRNA gene was amplified by PCR. The sequences of primers used for amplification were 5′-AGAGTTT GATCCTGGCTCAG-3′ and 5′-AAGGAGGTGAT CCAA/GCCGCA-3′, corresponding to positions 8 to 27 and 1521 to 1540, respectively, in the 16*S* rRNA sequence of *E. coli* (Brosius et al. 1978). The 1.5-kb PCR product was directly sequenced by the dideoxynucleotide chain termination method using a DNA sequencer (model 373A; Applied Biosystems, Foster City, CA, USA). Multiple alignments of the sequence were performed, nucleotide

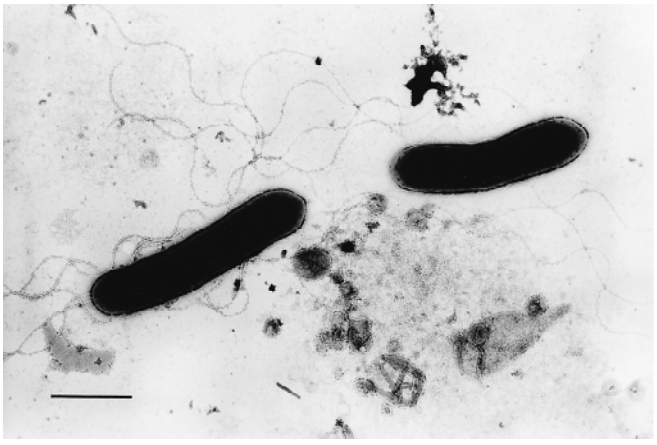

Fig. 1. Electron photomicrograph of negatively stained cells of strain YN-2000 grown at pH 10 showing peritrichous flagellation. *Bar* 1 μm

Table 1. Characteristics of the strain YN-2000 and *Bacillus cohnii* DSM 6307^T

Characteristic[a]	Strains YN-2000	*B. cohnii* DSM 6307^T
Color of colonies	White	White
Form	Rods	Rods
Motility	+[b]	+
Flagella	Peritrichous	Peritrichous
Oval spore	+	+
Gram stain	+	+
Catalase	+	+
Oxidase	+	+
Growth at pH 7	+	+
Aminopeptidase activity	−	−
Splitting of MUG	+	+
Growth at:		
10°C	−	−
47°C	+	+
49°C	−	−
Hydrolysis of:		
Casein	+	+
Gelatin	−	−
Starch	+	+
DNA	−	−
Pullulan	+	+
Hippurate	+	+
Tweens 20, 40, 60, and 80	+	+
Growth in:		
0%, 3%, 5%, 7%, 8%, 10%		
11%, 12%, 13% NaCl	+	+[c]
14% NaCl and up	−	−[c]
Deamination of		
phenylalanine	−	−
Reduction of NO_3 to NO_2	+	+
Dap	−	−
The major isoprenoid quinone	MK-7	MK-7
G + C mol%	35.8	35.8[c]

[a] All tests except the temperature range test and growth at pH 7 were performed at 30°C, pH 10
[b] +, positive; −, negative
[c] Tested in this study
All characteristics except growth in 1%–20% NaCl and G + C mol% of *Bacillus cohnii* are from Spanka and Fritze (1993)

Table 2. Fatty acid composition of total membrane lipid extracts from alkaliphilic *Bacillus* species and *Bacillus subtilis*

Fatty acids	Strain YN-2000 pH 7	Strain YN-2000 pH 10	*B. cohnii* DSM 6307^T pH 7	*B. cohnii* DSM 6307^T pH 10	*B. subtilis* IAM 1026 pH 7	*B. alcalophilus* JCM 5262^T pH 10
$isoC_{14:0}$	1.1	0.7	1.2	2.0	1.8	0.6
$C_{14:0}$	0.2	0.3	0.3	0.4	0.3	0.5
$isoC_{15:0}$	18.4	29.8	20.5	25.9	29.1	31.8
$anteisoC_{15:0}$	24.2	18.5	27.4	34.6	37.9	42.9
$C_{15:0}$	0.1	0.1	0.4	0.2	ND	0.3
$isoC_{16:0}$	4.8	2.9	4.9	5.3	4.9	1.0
$isoC_{16:1}$	0.5	0.1	0.6	1.3	0.1	0.2
$C_{16:0}$	1.8	2	2.3	0.9	3.2	2.1
$C_{16:1}$	4.9	2.3	5.1	3.1	0.2	1.5
$isoC_{17:0}$	5.7	11.5	6.2	4.4	11.2	4.3
$anteisoC_{17:0}$	19.2	14.6	16.8	10.0	9.6	11.2
$isoC_{17:1}$	9.1	10.8	8.7	6.9	0.3	1.7
$C_{17:0}$	0.9	0.6	0.6	0.2	ND	ND
$anteisoC_{17:1}$	4.3	2.7	3.8	4.1	0.2	0.9
$C_{18:0}$	0.7	0.8	0.4	ND	0.2	ND
$C_{18:1}$	0.3	ND	0.4	0.1	ND	ND
Others	3.7	2.9	0.9	0.7	1.1	0.4
Total unsaturated	19.1	15.9	18.6	15.5	0.8	4.3
iso-branched	39.6	55.8	42.1	45.8	47.4	39.6
anteiso-branched	47.7	35.8	48.0	48.7	47.7	55.0
Ratio anteiso:iso	1.20	0.64	1.14	1.06	1.01	1.39
Total branched	87.3	91.6	90.1	94.5	95.1	94.6
Straight saturated	3.7	3.8	4.0	1.7	3.7	2.9

ND, none detected

These results represent averages of four experiments with two independent preparations of cells grown at the indicated pH

substitution rates (K_{nuc} value) were calculated, and a neighbor-joining phylogenetic tree (Kimura 1980; Satiou and Nei 1987) was constructed using the CLUSTAL W program (Thompson et al. 1994). The similarity values of the sequences were calculated using the GENETYX computer program (Software Development, Tokyo, Japan).

DNA–DNA hybridization

The level of DNA–DNA relatedness was determined fluorometrically by the method of Ezaki et al. (1989) using photobiotin-labeled DNA probes and black microplate.

Electron microscopy

Electron microscopic observations under a transmission electron microscope (TEM) and scanning electron microscope (SEM) were performed as previously described (Ikeda et al. 1994; Yumoto et al. 1998a,b).

Results and discussion

Phenotypic and chemotaxonomic characteristics

Strain YN-2000 is a Gram-positive, aerobic rod with cells that were 0.6–1 by 1.5–5.5 μm long and produced subterminally located ellipsoidal spores. The cells were peritrichously flagellated (Fig. 1), and the strain was positive for oxidase and catalase reactions. The strain did not contain DAP in its cell walls. Phenotypic characteristics of strains YN-2000 and *B. cohnii* are shown in Table 1. A comparison of the physiological and biochemical characteristics of these two strains showed that they are very similar to each other. In addition to the taxonomic characteristics listed in Table 1, the characteristics of growth temperature at pH 7 and pH 10 and carbohydrate metabolism at pH 10 of strain YN-2000 were studied. When strain YN-2000 grew at pH 10, the strain was able to grow from 10°C to 47°C, whereas when the strain grew at pH 7, the growth range was 15° to 45°C. The strain YN-2000 produced acid from D-glucose, D-fructose, D-mannose, mannitol, trehalose, maltose, and cellobiose but not from lactose, melibiose, raffinose, sorbitol D-arabinose, *myo*-inositol, glycerol, and galactose. The DNA G + C content of strain YN-2000 was 35.8 mol%. The major isoprenoid quinone detected in strain YN-2000 was menaquinone-7.

16*S* rRNA sequence analysis and DNA–DNA hybridization

The 16*S* rRNA gene DNA of strain YN-2000 was sequenced to determine its phylogenetic position. The almost complete 16*S* rRNA sequence of strain YN-2000, consisting of 1503 nucleotides, was compared with the sequence of 17 alkaliphilic *Bacillus* strains and 10 neutrophilic species belonging to several groups of genus *Bacillus* as well as its related taxa (Fig. 2). Based on the phylogenetic analysis, strain YN-2000 was closest to *Bacillus cohnii*, with a high bootstrap value of 1000. The 16*S* rRNA sequence similarity of strain YN-2000 to *Bacillus cohnii* was 99.6%. These results show that strain YN-2000 should be identified as *Bacillus cohnii*.

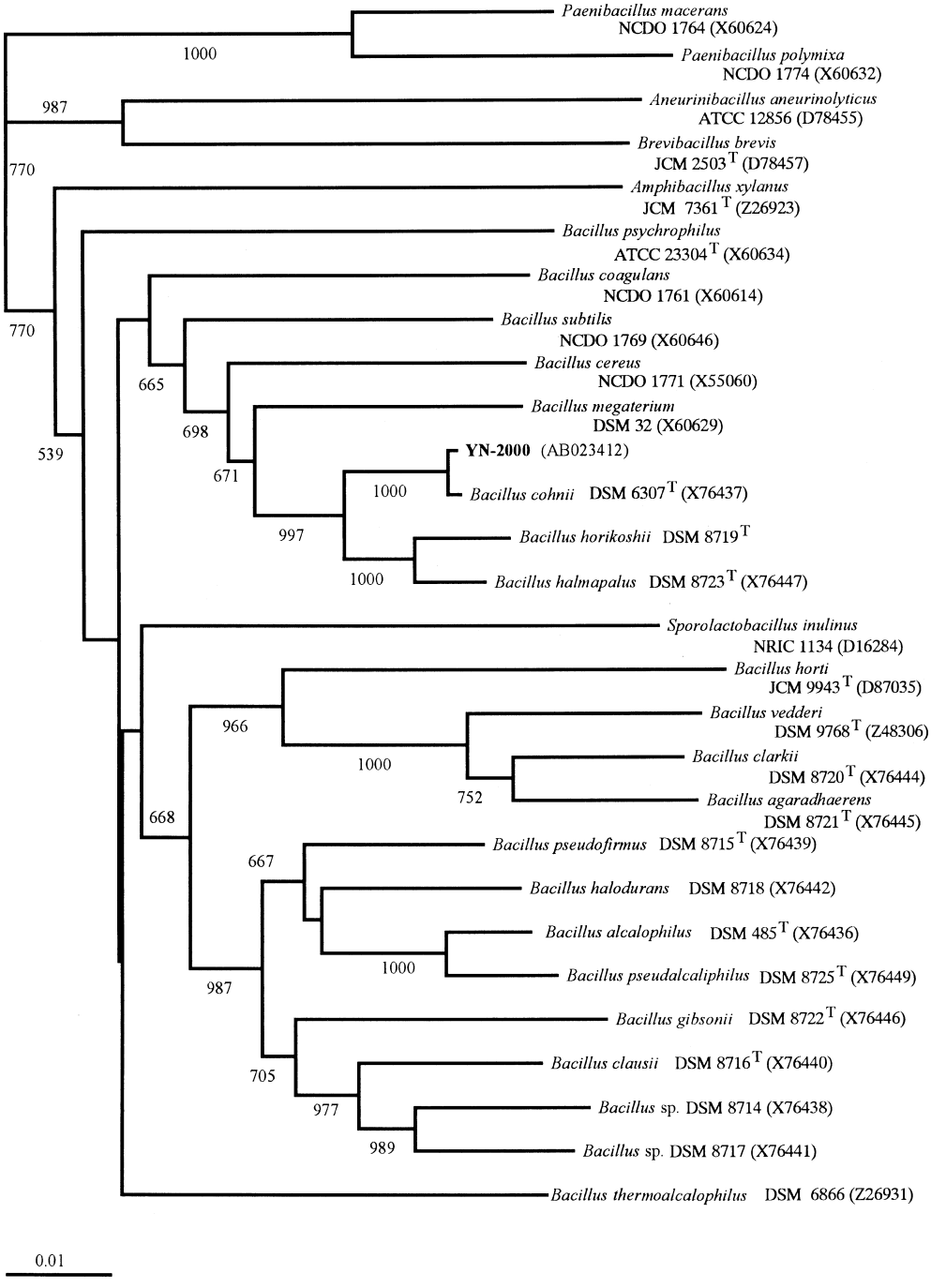

Fig. 2. Phylogenetic tree derived from 16*S* rRNA sequence data of strain YN-2000. *Numbers* are bootstrap values greater than 500. Bar 0.01 K_{nuc}

Based on the results observed, the level of DNA–DNA relatedness between strain YN-2000 and *Bacillus cohnii* was estimated. From those results, the DNA relatedness between the two strains was 100%. It was confirmed that strain YN-2000 belongs to *Bacillus cohnii*.

Fatty acid composition

The whole-cell fatty acid of strain YN-2000 grown at pH 10 consisted of major fatty acids, iso-$C_{15:0}$ (29.8%), anteiso-$C_{15:0}$ (18.5%), iso-$C_{17:0}$ (11.5%), anteiso-$C_{17:0}$ (14.6%), and iso-$C_{17:1}$ (10.8%) (Table 2). The fatty acid composition of *B. cohnii* DSM 6307^T grown at pH 10 was also analyzed by using the same medium and the same incubation and growth conditions. Although the fatty acid composition of stain YN-2000 was very similar to that of *B. cohnii* qualitatively, they were slightly different quantitatively. The contents of anteiso-$C_{15:0}$ and iso-$C_{17:0}$ were 18.5% and 11.5%, respectively, in strain YN-2000 at pH 10, whereas in the case of *B. cohnii* the contents of these fatty acids were 34.6% and 4.4%, respectively. A comparison of the difference in iso-$C_{17:0}$ content between the cells grown at pH 7 revealed that the fatty acid content of strain YN-2000

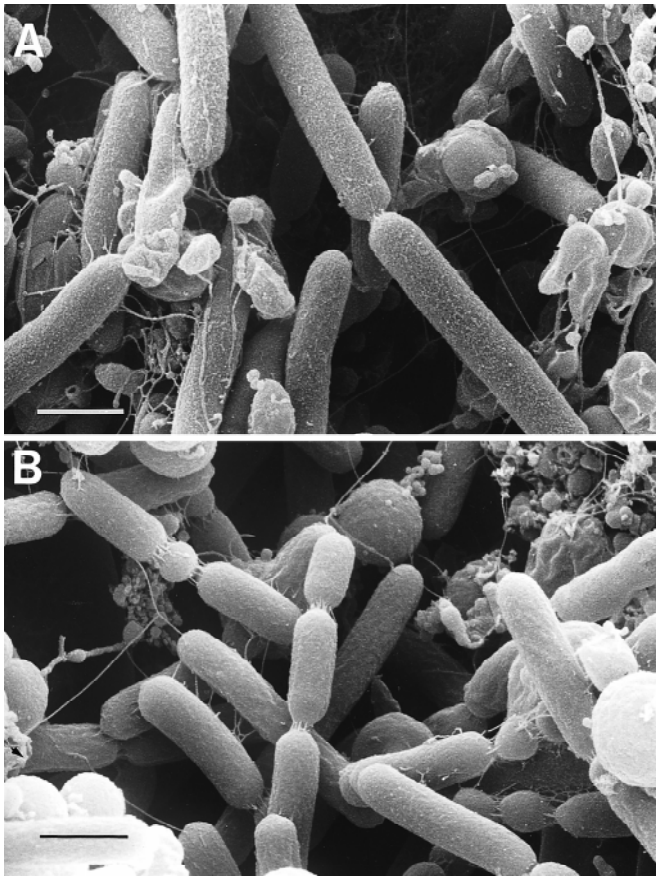

Fig. 3A,B. Scanning electron photomicrograph of strain YN-2000 grown at (**A**) pH 10 and (**B**) pH 7. In the cells grown at pH 10, the surface aspect was rougher and the cell shape was longer compared with cells grown at pH 7. *Bar* 1 μm

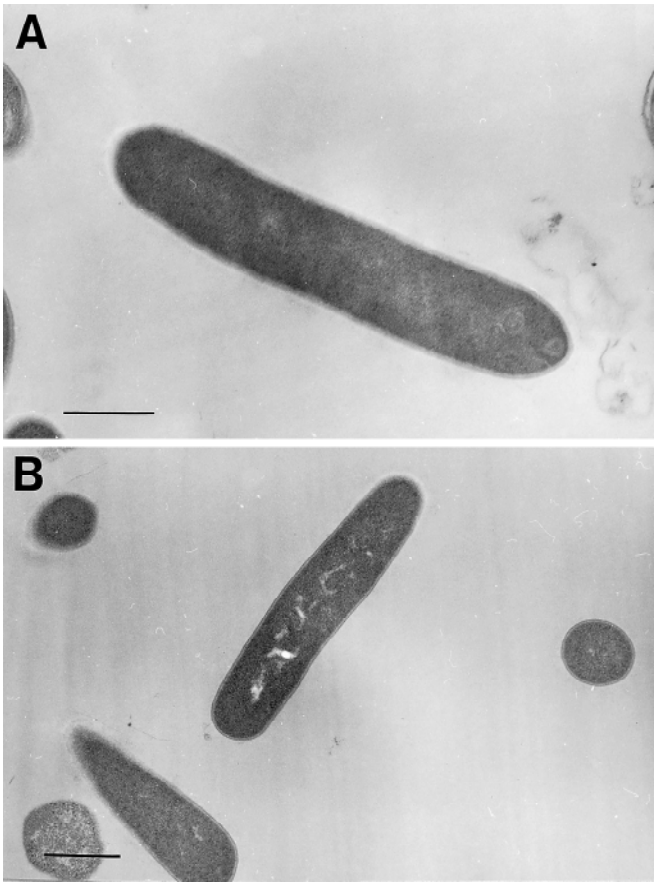

Fig. 4A,B. Electron photomicrograph of ultrathin section of strain YN-2000 cells grown at (**A**) pH 10 and (**B**) pH 7. The surface layer of the cells grown at pH 10 (39 nm) was thicker than that of the cells grown at pH 7 (17 nm). *Bar* 0.5 μm

increased from 5.7% to 11.5% with pH increase, whereas that of *B. cohnii* was decreased slightly, from 6.2% to 4.4%. These differences might be commonly observed within the same species.

Strain YN-2000 contained a larger amount of unsaturated fatty acids compared with *Bacillus subtilis* and the obligate alkaliphile, *Bacillus alcalophilus*, regardless of its culture pH (see Table 2). When the cells were grown at pH 10, the unsaturated fatty acid content and anteiso-/iso-branched fatty acid ratio became lower than those at pH 7. These results suggest that membrane fluidity becomes higher with decreasing culture pH, which is the opposite of the effect observed in a previous study (Clejan et al. 1986). Although strain YN-2000 is a facultative alkaliphilic strain, the fatty acid composition more closely resembled that in the obligate alkaliphilic strains in the previous study (Clejan et al. 1986). These differences might mean that the mechanism of adaptation to an alkaline environment differs among strains of alkaliphilic *Bacillus*.

Electron microscopic observation

In the cells of strain YN-2000 grown at pH 10, the cell-surface aspect appeared rougher and the cell shape appeared longer in the case of SEM observation (Fig. 3). The observation that the cells became longer in an alkali medium is also reported in other alkaliphilic strains (Sturr et al. 1994; Aono 1995). Although it was difficult to discriminate the components of the surface layer, especially in the case of the cells grown at pH 10, the surface layer of the cells grown at pH 10 was thicker than that of cells grown at pH 7 (Fig. 4). The thicker surface layer at pH 10 versus pH 7 might be attributed to proton trapping for producing a lower pH region on the surface of the cell membrane. That walls of the cells grown at pH 10 were thicker than those grown at neutral pH was also observed in the alkaliphilic strain C-125 (*Bacillus halodurans*) (Aono et al. 1995). These structural, quantitative, and qualitative changes in the cell surface might be related to the ability to grow in an alkaline environment.

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