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The formation and structures of cystites of *Arthrobacter ureafaciens* NRIC 0157^T induced by antibiotics

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The cystite formation of *Arthrobacter ureafaciens* NRIC 0157^T was induced by some antibiotics, and the addition of tetracycline at a lag phase was effective for cystite formation in YPM liquid medium. Cystites differed from vegetative cells in the cell wall structure, protein content, and water content. Furthermore, the characteristics and structures of cystites induced by tetracycline were similar to those of cystites produced by nutritional imbalance in CT medium. Consequently, various triggers would induce cystite formation. It is interesting that cystite formation was found in part of the *Arthrobacter* strains and seemed to correlate with the type of peptidoglycan.

Key Words—*Arthrobacter*; *Arthrobacter ureafaciens*; cell wall; cystites

Introduction

The members of the genus *Arthrobacter* generally inhabit soils and exhibit morphological changes of the cells, cocci-rods-cocci, during the life cycle (Claus and Berkeley, 1986). Apart from the normal life cycle, the formation of cystites has been described (Jensen, 1934; Stevenson, 1963). Duxbury et al. (1977) reported that the walls of *Arthrobacter globiformis* NCIB 10683 were thicker in cocci than in rods, the walls in cystites varied in thickness, and the chemical components of polysaccharides of cocci, cystites, and rods differed from one another. However, Duxbury et al. (1977) did not separate cystites clearly from vegetative cells, and the cultivated cells were not suitable for the

study of the cell components of cystites because cystites and vegetative cells (cocci and rods) would be mixed in preparations.

Previously, Tanaka et al. (2000) reported a design of a defined medium, CT liquid medium, for the cystite formation of *Arthrobacter ureafaciens* NRIC 0157^T, and the definition of cystites that are drumstick-shaped and oval cells, Gram-negative by the KOH reaction, and in a range of 1.5 to 3.5×1.5 to 7 µm in cell size. It is interesting that the cystites were not produced during the normal growth in YPM liquid medium (Tanaka et al., 2000). Furthermore, cystite formation resulted from an imbalance growth affected by the concentration of K⁺ and Mg²⁺ in CT liquid medium. Moreover, cystites were sometimes produced on other media, such as asparagine agar and glucose agar (Jensen, 1934; Tanaka et al., 2000). Consequently, other factors leading to the imbalance growth and the cystite formation of *A. ureafaciens* NRIC 0157^T would be expected, and antibiotics, therefore, were reminded because they are known to act as bacteriostatic or bactericidal agents (Madigan et al., 2000).

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^T: Indicates the type strain of a species.

This paper deals with the effect of antibiotics on the cystite formation of *A. ureafaciens* NRIC 0157^T and the structures of cell walls and the intracellular structures of cystites and vegetative cells. A correlation between the imbalance growth and the cystite formation of *A. ureafaciens* NRIC 0157^T is discussed.

Materials and Methods

Bacterial strains. The following strains were used in this study: *Arthrobacter agilis* IAM 14192 (IAM: IAM Culture Collection, Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo 113-0032, Japan); *A. atrocyaneus* IAM 12339^T; *A. aurescens* NRIC 0150^T (NRIC: NODAI Culture Collection Center, Tokyo University of Agriculture, Tokyo 156-8502, Japan); *A. citreus* NRIC 1858^T; *A. creatinolyticus* JCM 10102^T (JCM: Japan Collection of Microorganisms, The Institute of Physical and Chemical Research (RIKEN), Wako 351-0198, Japan); *A. crystallopoietes* JCM 2522^T; *A. cumminsii* DSM 10493^T (DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany); *A. globiformis* NRIC 0151^T; *A. histidinolorans* NRIC 0152^T; *A. ilicis* IFO 15514^T (IFO: Institute for Fermentation, Osaka, Osaka 532-8686, Japan); *A. mysorens* IAM 14869^T; *A. nicotianae* NRIC 0153^T; *A. nicotinovorans* IFO 15511^T; *A. oxydans* NRIC 0154^T; *A. pascens* IFO 12139^T; *A. polychromogenes* NRIC 0155^T; *A. protophormiae* IFO 12128^T; *A. ramosus* IAM 12344^T; *A. sulfureus* IFO 12678^T; *A. uratoxydans* IFO 15515^T; *A. ureafaciens* NRIC 0157^T; *A. woluwensis* DSM 10495^T; *Arthrobacter* sp. JCM 1339; *Nocardioides simplex* NRIC 1841^T; and *Terrabacter tumescens* NRIC 0156^T. Also, the following strains were used for controls: *Celulomonas biazotea* NRIC 0158^T; *Corynebacterium glutamicum* NRIC 0159^T; *Curtobacterium citreum* NRIC 1859^T; *Micrococcus luteus* CCM 169^T (CCM: Czech Collection Microorganisms, Brno, Czech Republic); *M. lylae* CCM 2693^T; *Kocuria rosea* CCM 679^T; *K. varians* CCM 884^T; *Dermacoccus nishinomiyaensis* CCM 2140^T; and *Stomatococcus mucilaginosus* CCM 2147^T.

Culture media. YPM liquid medium (Tanaka et al., 2000) was used for a basal medium and was composed of 1.0% yeast extract (Difco Laboratories, Detroit, MI, USA); 1.0% peptone (Mikuni Kagaku Sangyo Ltd., Tokyo, Japan), 0.075% K₂HPO₄, 0.075% KH₂PO₄, 0.009% MgSO₄·7H₂O, and 0.009% CaCl₂·2H₂O, and

pH was adjusted to 7.0 with NaOH. CT liquid medium (Tanaka et al., 2000) was composed of 2.0% D-glucose, 0.28% NH₄H₂PO₄, 0.136% K₂HPO₄, 0.136% KH₂PO₄, 0.0005% MgSO₄·7H₂O, and 0.0007% CaCl₂·2H₂O, and pH was adjusted to 7.5 with NaOH. Nutrient agar was used for viable cell counts (Tanaka et al., 2000), and was composed of 1.0% beef extract (Difco Laboratories), 1.0% peptone (Mikuni Kagaku Sangyo Ltd.), 0.5% NaCl, and 1.5% agar. The pH was adjusted to 7.0 with NaOH.

Cultivation. Strains were aerobically precultured in YPM liquid medium with 30 ml L-form test tubes or 500 ml Erlenmeyer flasks at 28 or 37°C. Cells at a late logarithmic phase were collected by centrifugation at 1,600×g for 10 min at 20°C and washed twice with saline. The washed cells were suspended in saline, and the suspension was inoculated with 1% (v/v) into fresh YPM liquid medium, CT liquid medium, or other liquid media with a pipette. The liquid media were aerobically incubated at 28 or 37°C for 1 or 2 days up to full growth.

Determination of bacterial growth. Bacterial growth in liquid media was determined by absorbance at 660 nm with a single-beam spectrophotometer (NO-VASPEC II, Amersham Pharmacia Biotech, Tokyo, Japan).

Investigation of cell shapes. Cell shapes were investigated with a phase-contrast microscope (BX-50, oil immersion lens, Olympus, Tokyo, Japan). The change of cell shapes was monitored at various intervals during the growth.

Determination of the Gram reaction by the KOH reaction. An incubated liquid medium was centrifuged at 1,600×g for 10 min, and precipitates were used to detect the KOH reaction (Powers, 1995).

Cell counts. A total cell number was determined with a Thoma-Zeiss counting chamber, and a viable cell number was determined by the number of colonies appearing on nutrient agar plates as previously described (Tanaka et al., 2000).

Scanning electron microscopy. Cells cultivated in liquid media were collected by centrifugation at 1,500×g for 10 min at room temperature and washed once with saline. The cells were suspended in saline and collected by centrifugation. The resulting cells were then suspended in 1 ml of 2% glutaraldehyde and shaken for 1 h. Further, the cells were centrifuged and washed with 1 ml of 0.1 M sodium phosphate buffer (pH 7.4) with shaking for 1 h. The cells were col-

lected by centrifugation and fixed in 1 ml of 1% OsO₄ with shaking for 3 h. The fixed cells were collected by centrifugation and dehydrated by ethanol. The ethanol concentration was increased in order of the following concentrations: 30, 50, 70, 80, 90, and 95% with shaking for 20 min in each concentration, and at 100% with shaking for 30 min. The cells were centrifuged and suspended in 100% ethanol for 30 min again. The dehydrated cells were centrifuged and suspended in *iso*-amyl acetate with shaking for 30 min. The cells were collected by centrifugation and dried with a critical point dryer (HCP-2, Hitachi Ltd., Tokyo, Japan). Dried specimens were shadowed with gold and palladium by the use of a sputter coating (E102, Hitachi Ltd.), and observed with a scanning electron microscope (S-4000, Hitachi Ltd.).

Transmission electron microscopy. Cells cultivated in liquid media were collected by centrifugation at 1,500×*g* for 10 min at room temperature, and washed once with saline. The cells were suspended in saline and collected by centrifugation. The washed cells were fixed with 2% glutaraldehyde and 1% OsO₄ and dehydrated by the procedure described above. The dehydrated cells were centrifuged, suspended in a mixture of ethanol–propylene oxide (50:50) for 20 min, then centrifuged again, followed by suspension in 98% propylene oxide. The cells were collected by centrifugation and suspended in a mixture of propylene oxide–Spurr epoxy (50:50) for 3 h. They were collected by centrifugation, and Spurr epoxy was dropped on pellets. The Spurr epoxy consisted of 10 ml of ERL-4206, 4 ml of DER 736, 26 ml of NSA, and 0.4 ml of S-1 (EM-Spurr set, Nisshin EM Ltd., Tokyo, Japan). Spurr epoxy containing the cells was polymerized for 8 h at 70°C. Ultrathin sections were prepared by the use of a glass knife made with an LKB knife maker (LKB Ltd., Bromma, Sweden) and with an LKB Ultratome (LKB Ltd.). They were stained with 1% uranyl acetate for 40 min, then with 1% Pb-citrate for 3 min. The sections were observed with a transmission electron microscope (JEM-100CX, JEOL Ltd., Tokyo, Japan).

Separation of cystites from vegetative cells. Cells cultivated in CT liquid medium or other liquid media were centrifuged at 2,500×*g* for 15 min at 4°C and suspended in 1/20 volume of saline. Cystites in the suspension were separated by density-gradient centrifugation. About 15 ml of 50% sucrose solution was added in a 50 ml centrifuge tube (40 mm in diameter), and 30% sucrose solution was then added carefully on

a 50% sucrose solution without mixing. Five milliliters of the suspension of cells was added on a 30% sucrose solution in a centrifuge tube. The centrifuge tubes were centrifuged with a swing-rotor at 400×*g* for 20 min at room temperature, and the 30% sucrose solution was carefully removed with a pipette after centrifugation. Cystites of *A. ureafaciens* NRIC 0157^T produced in liquid media were precipitated at the bottom of a centrifuge tube, and part of the cystites was still suspended in the 50% sucrose solution. The cystites suspended in the 50% sucrose solution were again collected by centrifugation (1,600×*g*, 15 min) at 4°C and washed with saline. Cystites left in the suspension were separated three times more in the same manner as described above.

Analyses of cell components. Peptidoglycans and cell wall components were prepared and determined by the method of Komagata and Suzuki (1987). Cells cultivated in liquid media were collected by centrifugation and disrupted by sonication. Specimens after sonication and centrifugation (12,000×*g*, 60 min) were regarded as a fraction of crude cell walls. A ratio of peptidoglycans to cell walls was determined by a quantity of amino acids in crude cell walls, and a ratio of peptidoglycans to whole cells was determined by a quantity of D-glutamic acid in freeze-dried cells. Freeze-dried cells were prepared similarly by the method of Komagata and Suzuki (1987) except for sonication. A quantity of D-glutamic acid was determined by high-performance liquid chromatography (HPLC) (TOSOH CCP&8010 series, Tosoh Corp., Tokyo, Japan) with a MCIGEL CRS10W column (4.6×50 mm, Mitsubishi Chemical Corp., Tokyo, Japan) and a UV-8010 detector (Tosoh Corp.). The eluent was 2 mM CuSO₄, and the column was kept at 35°C. The type of peptidoglycan of *Arthrobacter* species was referred to the papers of Schleifer and Kandler (1972), Claus and Berkeley (1986), and Funke et al. (1996).

Polysaccharides in crude cell walls were prepared by the sonication-SDS method (Takeuchi and Yokota, 1989). Neutral sugars in polysaccharides were determined by HPLC (Shimadzu LC-10A series, Shimadzu Corp., Kyoto, Japan) with an Asahipak NH2P-50 4E column (4.6×250 mm, Showa Denko K.K., Tokyo, Japan) and an RID-10A detector (Shimadzu Corp.). An eluent was composed of acetonitrile–distilled water–phosphoric acid (80:19:1, v/v), and the column was kept at 40°C. A ratio of polysaccharides to cell walls was determined by a quantity of neutral sugars in

crude cell walls.

The protein content in whole cells was determined by using freeze-dried cells and the Lowry method (Lowry et al., 1951).

The water content of cells was determined by using the weight of the wet cells and the dried cells.

Quinones and cellular fatty acids were determined by the methods of Komagata and Suzuki (1987).

Antibiotics. The following antibiotics were used: Ampicillin, cephalixin, chloramphenicol, cycloheximide, cycloserine, erythromycin, kanamycin, mitomycin C, nalidixic acid, streptomycin, and tetracycline. These antibiotics were filtered and added to YPM liquid medium at concentrations of 0.1 to 10 µg/ml, but cycloserine was added to YPM liquid medium at concentrations of 0.5 to 1,000 µg/ml.

Phylogenetic analysis based on 16S rDNA sequences. Published sequence data were obtained from GenBank and used for the phylogenetic analysis. The sequence data of *M. luteus* was that from IFO 16250, not from the type strain. The multiple alignments were performed by the program Clustal W (ver. 1.6) (Thompson et al., 1994). The distance matrixes for the aligned sequences were calculated by the two-parameter method of Kimura (1980) and expressed by K_{nuc} . The Neighbor-Joining method was used for constructing a phylogenetic tree (Saitou and Nei, 1987). The robustness for individual branches were estimated by bootstrapping with 1,000 replicates (Felsenstein, 1985).

Results

Effects of antibiotics on cell shapes of A. ureafaciens NRIC 0157^T

The growth of *A. ureafaciens* NRIC 0157^T was repressed to some extent by antibiotics used, and tetracycline, chloramphenicol, and erythromycin induced cystite formation. Drumstick-shaped cystites were induced by tetracycline at concentrations from 0.5 to 1.0 µg/ml, by chloramphenicol at concentrations from 1.5 to 2.5 µg/ml, and by erythromycin at a concentration of 0.5 µg/ml in YPM liquid medium. Oval cystites were induced by erythromycin at concentrations from 0.8 to 5.0 µg/ml in YPM liquid medium. Cystites induced in YPM liquid medium containing 0.8 µg/ml of tetracycline were similar to those produced in CT liquid medium in cell shape. Tetracycline, chloramphenicol, and erythromycin induced the formation of drumstick-

shaped or oval cells at the higher concentration, but these cells were not regarded as cystites because the size of the cells was much smaller compared with the real cystites. YPM liquid medium containing 0.8 µg/ml of tetracycline was named YPM-Tc liquid medium and was used throughout.

It is interesting that a V form resulting from snapping cell division and cystite formation were induced by cycloserine at concentrations from 50 to 100 µg/ml, and rods and cystites were shown at concentrations from 200 to 300 µg/ml in YPM liquid medium. Furthermore, elongated cells and cystites were induced by cephalixin at a concentration of 0.5 µg/ml, and mitomycin C at concentrations from 0.8 to 1.5 µg/ml. However, a ratio of cystites to vegetative cells was small in these three antibiotics. Ampicillin, cycloheximide, kanamycin, nalidixic acid, and streptomycin did not induce interesting cell shapes. Vegetative cells and cystites induced by antibiotics are shown in Fig. 1.

Cystites induced by tetracycline, chloramphenicol, and erythromycin in YPM liquid medium were Gram-negative by the KOH reaction. Cystites became viscous by 3% KOH solution, but the lysis of cells was not found microscopically.

Effects of tetracycline on the growth and cystite formation of A. ureafaciens NRIC 0157^T

The cells of *A. ureafaciens* NRIC 0157^T grown in YPM-Tc liquid medium swelled at terminals and turned to cystites (Fig. 2). The absorbance of YPM-Tc liquid medium at a 24-h cultivation was about 0.2 to 0.3, whereas the absorbance of YPM liquid medium was about 2 in the same manner (Fig. 3). Tetracycline repressed the growth. The total cell number was 7.7×10^8 cells/ml, and the viable cell number was 6.7×10^6 cells/ml in YPM-Tc liquid medium after a 24-h cultivation when 2.6×10^8 cells/ml were inoculated initially on this medium. In contrast, the total cell number was 3.4×10^{10} cells/ml, and the viable cell number was 1.1×10^{10} cells/ml in YPM liquid medium when the same inoculum was used.

Effects of the addition of tetracycline at different growth phases on the cystite formation of A. ureafaciens NRIC 0157^T

The growth of *A. ureafaciens* NRIC 0157^T was repressed when 0.8 µg/ml of tetracycline was added to YPM liquid medium at lag and logarithmic phases (Fig. 4). The cystite formation of *A. ureafaciens* NRIC 0157^T

Fig. 1. Cell shapes of *A. ureafaciens* NRIC 0157^T after one-day cultivation in YPM liquid medium containing antibiotics.

1: Vegetative cells in YPM liquid medium; 2: drumstick-shaped cystites induced by 0.8 $\mu\text{g/ml}$ of tetracycline; 3: drumstick-shaped cystites induced by 2.3 $\mu\text{g/ml}$ of chloramphenicol; 4: drumstick-shaped cystites induced by 0.5 $\mu\text{g/ml}$ of erythromycin; 5: oval cystites induced by 5.0 $\mu\text{g/ml}$ of erythromycin; 6: rods and drumstick-shaped cystites (arrow) induced by 0.5 $\mu\text{g/ml}$ of cephalixin; 7: rods and drumstick-shaped cystites (arrow) induced by 0.8 $\mu\text{g/ml}$ of mitomycin C; 8: V-form and drumstick-shaped cystites (arrow) induced by 100 $\mu\text{g/ml}$ of cycloserine; 9: rods and oval cystites (arrow) induced by 200 $\mu\text{g/ml}$ of cycloserine. A bar marker represents 5 μm .

was recognized by the addition of tetracycline at a start of cultivation, and after a 4-h cultivation (Fig. 5-1). However, the addition of tetracycline at logarithmic and stationary phases in YPM liquid medium did not result in cystite formation, but long rods and short rods were found (Fig. 5-2, 5-3). Cystite formation was induced by the addition of tetracycline only at a lag phase in YPM liquid medium.

Scanning electron microscopy

The surface of cystites appeared apparently to be similar to the surface of vegetative cells of *A. ureafa-*

ciens NRIC 0157^T (Fig. 6). It is interesting that swelling was observed around septa of cystites produced in CT liquid medium (Fig. 6-3), whereas the swelling around septa of vegetative cells and cystites in YPM-Tc liquid medium were not found (Fig. 6-1, 6-2).

Transmission electron microscopy

The thickness of cell walls of vegetative cells was from 20 to 30 nm when the cells of *A. ureafaciens* NRIC 0157^T were cultivated in YPM liquid medium. Furthermore, mesosomes and a filamentous nuclear region in vegetative cells were shown (Fig. 7-1). On

the other hand, cell walls of the cystites induced in YPM-Tc liquid medium were from 10 to 15 nm in thick. Mesosomes and a filamentous nuclear region were not found clearly (Fig. 7-2). Cystites produced in CT liquid medium were similar to cystites induced in YPM-Tc liquid medium in cell wall structures, and part of the cell walls of the cystite was separated from the cell. A filamentous nuclear region was found clearly, and mesosomes were not found in the cystites (Fig. 7-3).

Outer cell walls were not found in the cells cultivated in CT liquid medium (Fig. 8). Invagination was not found in cystites cultivated in YPM-Tc liquid medium.

Fig. 2. Changes of cell shapes of *A. ureafaciens* NRIC 0157^T cultivated in YPM-Tc liquid medium.

1: Cells at 0 h; 2: cells at 8 h; 3: cells at 12 h; 4: cells at 24 h. A bar marker represents 5 μ m.

Fig. 5. Cell shapes of *A. ureafaciens* NRIC 0157^T cultivated in YPM liquid medium after adding 0.8 μ g/ml of tetracycline at different growth phases.

1: Cystites induced by adding at start, 0 h (lag phase); 2: long rods induced by adding at 8 h (logarithmic phase); 3: short rods induced by adding at 16 h (stationary phase). A bar marker represents 5 μ m.

Chemical analysis of cell components

Peptidoglycans in the cell walls of vegetative cells of *A. ureafaciens* NRIC 0157^T were composed of alanine,

Fig. 3. Growth of *A. ureafaciens* NRIC 0157^T in YPM liquid medium and YPM-Tc liquid medium.

● YPM liquid medium; ○ YPM-Tc liquid medium.

Fig. 4. Growth of *A. ureafaciens* NRIC 0157^T cultivated in YPM liquid medium after adding 0.8 μ g/ml of tetracycline.

○ Addition at start, 0 h; △ addition at 4 h; ■ addition at 8 h; ● addition at 16 h.

Fig. 6. Photos of a vegetative cell and cystites of *A. ureafaciens* NRIC 0157^T with scanning electron microscopy.

1: A vegetative cell grown in YPM liquid medium; 2: a cystite produced in YPM-Tc liquid medium; 3: a cystite induced in CT liquid medium. Bar markers represent 0.5 μm .

glutamic acid, lysine, and threonine (approximately 4:1:1:1). Cystites induced in YPM-Tc liquid medium showed the same composition as the vegetative cells. Peptidoglycans of vegetative cells shared 70.4% of cell walls, whereas peptidoglycans of cystites shared 51.2% of cell walls when cystites were induced in YPM-Tc liquid medium (Table 1).

The major component of polysaccharides in the cell walls of vegetative cells was galactose, and the component of cystites induced in YPM-Tc liquid medium was glucose at a stationary phase after a 24-h cultivation. Polysaccharides shared 9.9% of cell walls of vegetative cells, and the polysaccharides shared 42.4% of cell walls of cystites induced in YPM-Tc liquid medium. Furthermore, the kinds of monosaccharides and the

Fig. 7. Ultrathin section representative of a vegetative cell and cystites of *A. ureafaciens* NRIC 0157^T.

1: A vegetative cell grown in YPM liquid medium; 2: a cystite induced in YPM-Tc liquid medium; 3: a cystite produced in CT liquid medium. Bar markers represent 200 nm. M: mesosome; N: nuclear region.

Fig. 8. Ultrathin section representative of a septum of a cystite of *A. ureafaciens* NRIC 0157^T.

An inner cell wall was invaginated, and swelling was observed around a septum. A bar marker represents 0.1 μm .

Table 1. The peptidoglycan of vegetative cells and cystites of *A. ureafaciens* NRIC 0157^T.

	Composition of peptidoglycan ^a							Peptidoglycan in cell wall (%)
	Thr ^b	:	Glu	:	Ala	:	Lys	
Vegetative cells (in YPM liquid medium)	1.1	:	1.0	:	3.6	:	0.9	70.4
Cystites (in YPM-Tc liquid medium)	1.2	:	1.0	:	3.7	:	1.0	51.2
Cystites (in CT liquid medium)	1.1	:	1.0	:	3.6	:	1.0	60.8

^a Molar ratio to glutamic acid.^b Thr: threonine; Glu: glutamic acid; Ala: alanine; Lys: lysine.Table 2. The polysaccharide in cell walls of vegetative cells and cystites of *A. ureafaciens* NRIC 0157^T at logarithmic and stationary phases.

	Component of polysaccharide		Polysaccharide in cell wall (%)
	Galactose	: Glucose	
Logarithmic phase (8-h cultivation)			
Vegetative cells (in YPM liquid medium)	1.0	: 0.3	9.2
Cystites (in YPM-Tc liquid medium)	1.0	: 3.0	22.8
Cystites (in CT liquid medium)	1.0	: 1.7	15.3
Stationary phase (24-h cultivation)			
Vegetative cells (in YPM liquid medium)	1.0	: 0.3	9.9
Cystites (in YPM-Tc liquid medium)	1.0	: 9.3	42.4
Cystites (in CT liquid medium)	1.0	: 9.0	35.8

total quantity of polysaccharides in cell walls of cells cultivated in YPM-Tc liquid medium were also different from those in YPM liquid medium at a logarithmic phase after an 8-h cultivation. The cell walls of cystites produced in CT liquid medium were similar to those induced in YPM-Tc liquid medium in the component. Data are shown in Table 2. Cell walls in the whole cells shared 4.8% in vegetative cells, 7.2% in cystites induced in YPM-Tc liquid medium, and 5.1% in cystites produced in CT liquid medium.

Furthermore, vegetative cells grown in YPM liquid medium contained 61.7 and 63.5% protein at lag and logarithmic phases, respectively. On the other hand, cystites induced in YPM-Tc liquid medium had a half-

amount of protein compared with that of vegetative cells. It is interesting that cells (long rods) grown in CT liquid medium had the same amount of protein as vegetative cells at a logarithmic phase, and only 41% protein was found in cystites produced in CT liquid medium after a 24-h cultivation at a stationary phase. Data are shown in Table 3.

The water content of vegetative cells, cystites induced in YPM-Tc liquid medium, and cystites produced in CT liquid medium was 73, 81, and 84%, respectively.

The major menaquinone of vegetative cells and cystites was MK-9(H₂), and the major cellular fatty acid was 12-methyl tetradecanoic acid (*anteiso*-C_{15:0}).

Table 3. The content of protein in dried vegetative cells and dried cystites of *A. ureafaciens* NRIC 0157^T at logarithmic and stationary phases.

	Content of protein (%)	
	Logarithmic phase	Stationary phase
Vegetative cells (in YPM liquid medium)	61.7	63.5
Cystites (in YPM-Tc liquid medium)	35.7	29.3
Cystites (in CT liquid medium)	60.9	41.0

Chemical compositions of cystites produced in CT liquid medium were similar to those in YPM-Tc liquid medium after a 24-h cultivation. Data are shown in Tables 1, 2, and 3.

Cystite formation of Arthrobacter strains induced by tetracycline in YPM liquid medium

A. aurescens NRIC 0150^T, *A. crystallopoietes* JCM 2522^T, *A. ilicis* IFO 15514^T, *A. nicotinovorans* IFO 15511^T, *A. oxydans* NRIC 0154^T, *A. pascens* IFO 12139^T, and *A. ramosus* IAM 12344^T formed cystites in YPM-Tc liquid medium as *A. ureafaciens* NRIC 0157^T did. *A. atrocyaneus* IAM 12339^T, *A. globiformis* NRIC 0151^T, and *Arthrobacter* sp. JCM 1339 were

Fig. 9. Cells of *Arthrobacter* strains cultivated in YPM liquid medium and YPM-Tc liquid medium. 1: Vegetative cells of *Arthrobacter ilicis* IFO 15514^T grown in YPM liquid medium; 2: drumstick-shaped cystites of *A. ilicis* IFO 15514^T induced in YPM-Tc liquid medium; 3: vegetative cells of *Arthrobacter oxydans* NRIC 0154^T grown in YPM liquid medium; 4: oval cystites of *A. oxydans* NRIC 0154^T induced in YPM-Tc liquid medium; 5: vegetative cells of *Arthrobacter nicotianae* NRIC 0153^T grown in YPM liquid medium; 6: vegetative cells of *A. nicotianae* NRIC 0153^T cultivated in YPM-Tc liquid medium; 7: vegetative cells of *Arthrobacter protophormiae* IFO 12128^T grown in YPM liquid medium; 8: vegetative cells of *A. protophormiae* IFO 12128^T cultivated in YPM-Tc liquid medium. A bar marker represents 5 μm.

also induced to form cystites by 5 µg/ml of tetracycline in YPM liquid medium, and *A. citreus* NRIC 1858^T and *A. polychromogenes* NRIC 0155^T formed cystites in YPM liquid medium containing 10 µg/ml of tetracycline. The cystites of these strains were drumstick-shaped or oval, and their cystites showed Gram-negative by the KOH reaction. *A. creatinolyticus* JCM 10102^T, *A. cummingsii* DSM 10493^T, *A. histidinolovorans* NRIC 0152^T, *A. mysorens* IAM 14869^T, *A. nicotianae* NRIC 0153^T, *A. protophormiae* IFO 12128^T, *A. sulfureus* IFO 12678^T, *A. uratoxydans* IFO 15515^T, and *A. woluwensis* DSM 10495^T produced no cystites, though their growth was repressed by tetracycline in YPM liquid medium. Moreover, *Cellulomonas biazotea* NRIC 0158^T, *Corynebacterium glutamicum* NRIC 0159^T, *Curtobacterium citreum* NRIC 1859^T, *Nocardioides simplex* NRIC 1841^T, *Terrabacter tumescens* NRIC 0156^T, *Micrococcus luteus* CCM 169^T, *M. lylae* CCM 2693^T, *Kocuria rosea* CCM 679^T, *K. varians* CCM 884^T, *Dermacoccus nishinomiyaensis* CCM 2140^T, and *Stomatococcus mucilaginosus* CCM 2147^T were not induced to form cystites by tetracycline. The cells of *A. ilicis* IFO 15514^T, *A. oxydans* NRIC 0154^T, *A. nicotianae* NRIC 0153^T, and *A. protophormiae* IFO 12128^T grown on YPM and YPM-Tc liquid medium are shown in Fig. 9.

The cells of *A. nicotianae* NRIC 0153^T grown in YPM liquid medium, YPM-Tc liquid medium, and CT liquid medium all showed the same structures (Fig. 10).

Phylogenetic relationship of the cystite formation among Arthrobacter species

Arthrobacter strains were grouped by 16S rDNA sequences into 4 clusters and 7 clusters consisting of a single strain (Fig. 11). Cystite-forming strains were placed in the *Arthrobacter globiformis* cluster, the *Arthrobacter oxydans* cluster, the *Arthrobacter ureafaciens* cluster, and three clusters consisting of a single strain: *Arthrobacter citreus*, *Arthrobacter atrocyaneus*, and *Arthrobacter crystallopoietes*. Strains grouped into the *Arthrobacter globiformis* cluster and the *Arthrobacter ureafaciens* cluster were induced to form drumstick-shaped cystites, and strains grouped into *Arthrobacter oxydans* cluster were induced to form oval cystites by tetracycline.

Fig. 10. Cell structure of *Arthrobacter nicotianae* NRIC 0153^T cultivated in YPM liquid medium, CT liquid medium, and YPM-Tc liquid medium.

1: A cell cultivated in YPM liquid medium; 2: a cell cultivated in YPM-Tc liquid medium; 3: a cell cultivated in CT liquid medium. Bar markers represent 0.2 µm.

Discussion

Antibiotics effected on the cell shape of *Arthrobacter* strains, and tetracycline was effective for cystite formation. The cystites of *A. ureafaciens* NRIC 0157^T were induced in YPM-Tc liquid medium, and the formation of cystites was induced only by the addition of tetracycline at a lag phase. This resulted from the unusual formation of cell walls and septa. The cell wall structures of the cystites differed from those of vegetative cells, and cystites were not produced by the addition of tetracycline at logarithmic and stationary phases. The cystites of *A. ureafaciens* NRIC 0157^T were produced not only by antibiotics, but also by the imbalance of the

Fig. 11. Phylogenetic relationships of the cystite-forming strains based on 16S rDNA sequences.

© Cystite-forming strains. The scale bar represents 2 nucleotide substitutions per 100 nucleotides. Numericals indicate the bootstrap values derived from 1,000 replications.

concentration of minerals in CT liquid medium. The cells cultivated in the latter medium swelled after the cell division of about three times (Tanaka et al., 2000). Cystites were unable to elongate and produce daughter cells. Therefore, cystite formation would be caused by unusual growth in CT liquid medium. Toyota and Kitahara (1972) reported that the formation of swollen cells of *Sporolactobacillus* strains was effected by the concentration of NaCl, and that the swollen cells resulted from the imbalance of elongation of cells and cell wall synthesis. Thus the cell shapes of bacteria

would be affected by several factors in environment.

The cell walls of the cystites of *A. ureafaciens* NRIC 0157^T induced in YPM-Tc liquid medium were thinner than those of vegetative cells. Furthermore, the cell wall structures of cystites induced in YPM-Tc liquid medium were the same as the structures of cystites produced in CT liquid medium. Krulwich and Pate (1971) reported the production of an outer cell wall on the formation of septa of vegetative cells of *A. crystallopoietes* ATCC 15481^T. The quantity of the peptidoglycans in the cell walls of *A. ureafaciens* NRIC 0157^T

decreased when vegetative cells turned to cystites, and outer cell walls were not found on cystites. Therefore the peptidoglycans of the cystites of *A. ureafaciens* NRIC 0157^T would not be produced normally. Cystites became viscous by the KOH reaction, but viscosity was not produced by the lysis of cystites. Therefore the surface of cystites of *A. ureafaciens* NRIC 0157^T would be different from the surface of vegetative cells. The quantity and components of polysaccharides in the cell walls of cystites were much more different from those of vegetative cells. The cystites of *A. ureafaciens* NRIC 0157^T had much more polysaccharides in cell walls than vegetative cells did. The components of polysaccharides were reported to show a variety in the cell walls of *Arthrobacter* strains (Takeuchi and Yokota, 1989). Therefore the components of polysaccharides in cell walls would not correlate with cystite formation, and the production of polysaccharides of the cell walls of cystites would have an effect more distinctly on cystite formation and the KOH reaction than the production of peptidoglycans would.

In this study, the cystite formation of *A. ureafaciens* NRIC 0157^T was shown to be induced not only by tetracycline but also by chloramphenicol and erythromycin. Tetracycline and chloramphenicol induced the formation of drumstick-shaped cystites, and erythromycin induced the formation of oval cystites. The shapes of cystites would be effected by the mode of actions of antibiotics on the elongation of cells. It is interesting that cycloserine induced cystite formation, and snapping cell division was found at limited concentrations from 50 to 100 µg/ml. The snapping division was not found during the normal growth of *A. ureafaciens* NRIC 0157^T (Komagata et al., 1969). This may be a clue to make clear the mechanism of the cell division of *Arthrobacter* strains. Moreover, cephalixin and mitomycin C induced cystite formation.

Generally, tetracycline, chloramphenicol, and erythromycin were reported to inhibit the protein synthesis by binding on the 30S or 50S subunits of 70S ribosomes (Fernandez-Muñoz et al., 1971; Sarkar and Thach, 1968; Vazquez, 1966). Cycloheximide, kanamycin, and streptomycin are also reported to inhibit the protein synthesis, but they do not inhibit the growth of Gram-positive bacteria (Madigan et al., 2000). Cephalixin and cycloserine inhibit the cell wall synthesis, and mitomycin C inhibits DNA synthesis (Iyer and Szybalski, 1964; Morin et al., 1969; Neuhaus and Lynch, 1964). As mentioned above, antibiotics in-

fluence biosyntheses, and induced the cystite formation of *A. ureafaciens* NRIC 0157^T. Therefore various triggers would induce cystite formation. As reported in a previous paper (Tanaka et al., 2000), the concentration of K⁺ and Mg²⁺ induced the cystite formation of *A. ureafaciens* NRIC 0157^T in CT liquid medium, which would be one of the triggers, and also the superfluous consumption of D-glucose and NH₄⁺ for the growth (Tanaka et al., 2000). However, the superfluous consumption would not have an effect on cystite formation in CT liquid medium because cystites were produced in YPM-Tc liquid medium in which amounts of D-glucose and NH₄⁺ were negligible. In fact, D-glucose and NH₄⁺ were not detected in uncultured YPM-Tc liquid medium. Consequently, various triggers would disturb cellular constituents which would raise the imbalance of synthesis of the cell wall, the protein, and the DNA, resulting in cystite formation. Studies of the synthesis of intracellular structures may be important hints to solve the mechanism of cystite formation. Cystites differed clearly from vegetative cells in intracellular structures. Furthermore, protein was not produced so much for cystite formation, and cystites stored more water than vegetative cells did. They would keep water in cells more than vegetative cells because the cell walls of cystites kept much amounts of polysaccharides.

Arthrobacter species were classified into two groups by the type of peptidoglycan, A3α group and A4α group (Claus and Berkeley, 1986). Cystite-forming strains in YPM-Tc liquid medium were restricted to strains with A3α the same as those in CT liquid medium (Tanaka et al., 2000). Cystites produced in CT liquid medium and in YPM-Tc liquid medium were similar in shape and in chemical components with each other, i.e., drumstick-shaped and oval. *Arthrobacter* strains were grouped into a sizable number of clusters on the basis of 16S rDNA sequences, but cystite-forming strains were widely distributed in clusters in the genus *Arthrobacter*. The shapes of cystites are homogenous in each of the clusters. Moreover, the clusters were divided into those including the drumstick-shaped cystites forming strains and the oval cystites forming strains. Therefore the type of peptidoglycan and the shape of the cystites seemed to reflect phylogenetic relationships in *Arthrobacter* strains.

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