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A new rapid method of glycolate test by diethyl ether extraction, which is applicable to a small amount of bacterial cells of less than one milligram

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The glycolate test is a method to discriminate N-acyl groups of muramyl residue in peptidoglycan of bacterial cell walls by color reaction without purification of the cell walls. The glycolyl residue presents red purple color by heating with 0.02% 2,7-dihydroxynaphthalene (DON) dissolved in concentrated H_2SO_4. Instead of the previous column methods for quantitative analysis, a qualitative method by solvent works was developed to simplify and to miniaturize the analysis. In this method, solvents played two roles, removal of interfering materials and extraction of glycolic acid from the cell hydrolysates. Of several solvent systems tested, diethyl ether was studied in detail on such properties as the efficiency of glycolic acid extraction under several conditions, the ability of removing various interfering compounds, and the advantage on evaporation procedure of the solvent from extracts. DON reaction of the second diethyl ether extract from cell hydrolysate of "*Micromonospora nigra*" JCM 3328 showed a clear red purple color of a strong absorbance at 530 nm, which is the same as that of authentic glycolic acid. The solvent method was applied to 20 strains of typical actinomycete species whose acyl types have already been known (Uchida and Seino, 1997). All glycolate test positive strains showed the clear red purple color mentioned above, whereas acetyl type strains revealed no apparent color by the same procedures. Additional experiments indicated that the glycolate test could be determined with less than 1 mg of actinomycete cells by using a smaller amount of DON reagent and ordinary polypropylene tubes. The new method was discussed for advantages in the identification of actinomycetes and for possible applications to other fields.

Key Words——actinomycetes; bacterial cell wall; color test; 2,7-dihydroxynaphthalene (DON) reagent; glycolate test; microanalysis; N-acyl type of muramyl residues

N-Acyl type of muramyl residue in peptidoglycan is a stable and taxon-specific characteristic, which can be determined with whole cell hydrolysates by glycolate test (Uchida and Aida, 1977, 1979). Therefore this test has been extensively used for identification and classification of bacteria (Asano and Kawamoto, 1986; Collins et al., 1983; Kawamoto et al., 1981; Rainey et al., 1995; Schleifer and Stackebrandt, 1983; Stackebrandt et al., 1988; Tamura et al., 1994; Uchida and Seino, 1997; Yokota et al., 1993a, b). In these studies, column methods have been employed for quantitative determination of the acyl content in cells with ion-exchange resins of the cation and anion types (Uchida and Aida, 1977) or the singly anion type (Komagata and Suzuki, 1987; Uchida and Aida, 1984). The column method is considered to have some problems in its practice. The method needs glass columns of the described size and anion exchange resin of the acetyl type. Furthermore, successive elution procedures on the column(s) result in excess dilution of the acyl residue(s) in the sample solution for the purpose of full recovery of the compound(s). The method, therefore, had difficulty in determining the small amount of bacterial cells of less than 10 mg of dried cells. Moreover, it is indicated that such a quantitative analysis is not always necessary for identification of the acyl types (Uchida and Seino, 1997). Accumulated data show that the amino groups on the glucosaminyl and muramyl residues in the peptidoglycan are both acetylated or acylated with a glycolyl group only on the mu-

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ramyl part in restricted bacterial taxa, and they indicate that when much amount of glycolic acid is detected in the cell hydrolysate, it is essentially derived from muramyl residues in the peptidoglycan (Uchida and Aida, 1977, 1979). Considering this information, we tried to develop a simplified qualitative glycolate test of the acyl types of bacterial cells by means of solvent fractionation without resin column(s). This simple and rapid method is applicable to many strains for classification and identification of actinobacteria, using small amounts of cells of less than 1 mg.

Materials and Methods

Chemicals. Glycolic acid, sodium glycolate, and 2,7-dihydroxynaphthalene (2,7-naphthalenediol, abbreviated as DON below) were purchased from Tokyo Chemical Inc. (Tokyo, Japan). Diethyl ether (G grade), trifluoroacetic acid (G grade), and H_2SO_4 (G grade) are products of Kanto Chemical Inc. (Tokyo, Japan). All plastic microtest tubes used were Safe-Lock 1.5 ml (Eppendorf-Netheler-Hinz-GmbH, Hamburg, Germany) made of polypropylene.

Purification of DON. Some experiments were carried out with a purified DON. The purification method was as follows: Methanol (3 to 4 ml) was added by drops to 2 g of commercially available DON in a 100 ml flask to dissolve. Subsequently, approximately 60 ml of benzene was added to the solution slowly at room temperature until a small amount of crystals appeared. After a few days, the crystals were washed with benzene two times and dried.

Determination of glycolic acid. The concentration of glycolic acid was measured by a colorimetric method. Unless otherwise stated, the method used for the quantitative determination of glycolate was described in the previous papers (Uchida and Aida, 1977, 1984). Briefly, 2 ml of DON reagent (0.02% DON in concentrated H_2SO_4) was added to 0.1 ml of sample solution and kept at 100°C for 10 min. The reaction mixture was diluted with 1.9 ml of H_2SO_4, and measured by spectrophotometer at 530 nm.

Bacterial strains and culture conditions. Bacterial strains were obtained from IAM, IFO, and JCM culture collections. Some organisms from the personal stock of K. Uchida were also used (cultures with AKU numbers). Abbreviations: IAM, IAM Culture Collection, Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo, Japan; IFO, Institute for Fermentation, Osaka, Japan; JCM, Japan Collection of Microorganisms, RIKEN (The Institute of Physical and Chemical Research), Wako, Saitama, Japan.

The strains were cultured on a medium containing 10.0 g of soluble starch, 4.0 g of yeast extract (Difco Laboratories, Detroit, MI, USA), 4.0 g of Tryptone (Difco), 2.0 g of sucrose, and 15.0 g of agar in 1 L of distilled water (finally adjusted to pH 7.2) at 27°C except for *Pilimelia anulata* IFO 15533, which was cultured on Czapek's peptone agar. Bacterial cell mass was scraped from the plate after 3 days to 3 weeks culture. The cells with some attached agar were suspended in distilled water, heated, and the dissolved agar was removed by pipetting. They were occasionally treated by several washings with hot water. The resulting cell mass was lyophilized and subjected to glycolate analyses.

Equipment. The hydrolysis of cells and glycolate-DON reaction was carried out at 100°C in culture tubes with metal block bath EMG-2 (Tokyo Rikakikai Co., Ltd., Tokyo, Japan). The solvent extraction of glycolic acid from cell hydrolysates was done with an automatic mixer S-100 (Taitec Corp., Tokyo, Japan), or a microtube mixer MYT-360 (Tomy, Tokyo, Japan). Absorption spectra of glycolic acid were obtained with spectrophotometer UV-2100 (Shimadzu Corp., Kyoto, Japan).

Results

I. Physical and chemical properties of glycolic acid affecting the procedures in glycolate test

1. Effects of concentration of DON solution in H_2SO_4 to the color intensity in the test. Glycolic acid gives a clear red purple color after 100°C for 10 min with DON in concentrated H_2SO_4 (Uchida and Aida, 1977). The concentration of DON reagent that contains 20 mg of DON in 100 ml of H_2SO_4 has been used for full-color development of glycolic acid (Komagata and Suzuki, 1987; Uchida and Aida, 1977, 1984; Uchida and Seino, 1997). Experiments showed that the DON concentration could be reduced to 5 mg/100 ml concentrated H_2SO_4 without marked loss of color intensity (Fig. 1).

2. Quality of DON reagent during storage. During storage of DON reagent at room temperature, the reagent gradually became reddish in a few days without glycolic acid (data not shown). This coloring was effectively prevented for 5 weeks by keeping the reagent at 4°C in a capped bottle (Fig. 2). Color intensity yielded by the reaction with the reagent and glycolic acid was stable as seen in Fig. 2. An experiment using a DON reagent prepared with recrystallized DON showed almost the same blank values and reaction colors as those with the commercially available chemicals through the test period.

3. Extractability of glycolate by several solvent systems. Several solvent systems were tested to extract glycolic acid effectively from a water solution (Table

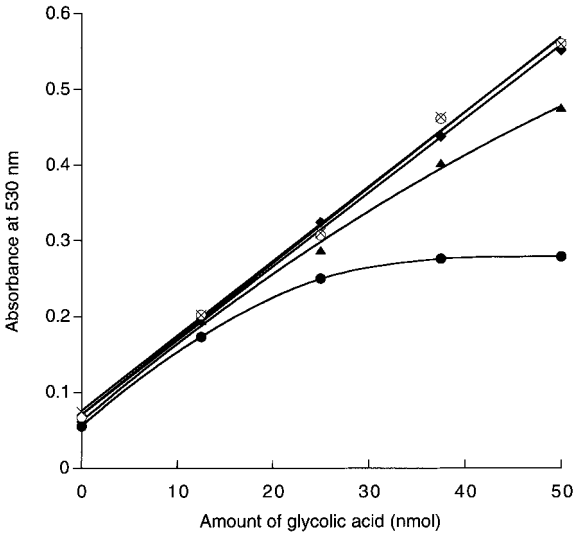

Fig. 1. Concentration of DON in DON reagent necessary for full color development of glycolic acid.

The amounts of DON in 100 ml of concentrated H_2SO_4: ●, 1 mg; ▲, 2 mg; ◆, 5 mg; ○, 10 mg; and ×, 20 mg.

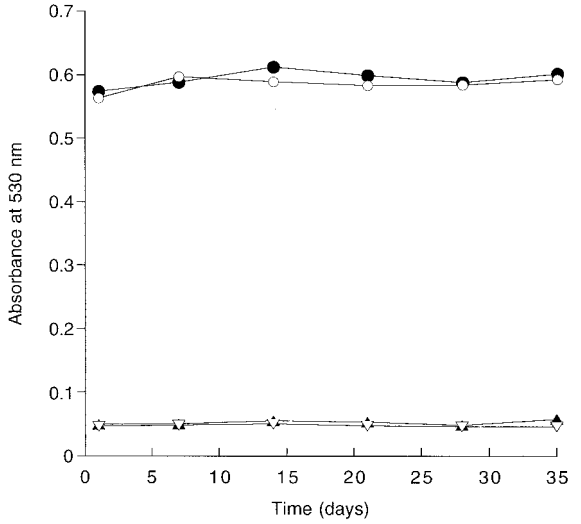

Fig. 2. Effects of storage of DON reagents at 4°C and purification of DON by recrystallization on the color reaction.

▲, nonpurified reagent (blank); ▽, purified reagent (blank); ●, nonpurified reagent with glycolic acid (50 nmol); ○, and purified reagent with glycolic acid (50 nmol).

1). Glycolic acid solution (0.2 ml) was vigorously shaken with 10 volumes of water-saturated diethyl ether, and the solvent was removed by means of standing in a draft or by using a small fan. The result showed that about 21% of the glycolate was transferred to the diethyl ether fraction. The yield was high enough to use. Although ethyl acetate had a higher ability to extract glycolate, it was not easily removed by evaporation from the extract.

Table 1. Extraction of glycolic acid by several solvent systems.

Solvents	Efficiency of extraction (%)
n-Hexane	0.9
n-Hexane-dichloromethane (2 : 1)	0.0
n-Hexane-diethyl ether (2 : 1)	0.8
Diethyl ether	20.8
Ethyl acetate	41.2

Two hundred nanomoles of glycolic acid in 0.2 ml of 1 N HCl were taken in a culture tube (16 mm $\phi \times 100$ mm) and extracted with 2.0 ml each of the above solvents (water-saturated) by vigorously shaking in a mixer for 1 min. After brief centrifugation, the solvent layer was carefully transferred, alkalized, and evaporated. The debris was dissolved in 0.1 ml of distilled water and the glycolate concentration determined by the DON reagent. The efficiency of extraction was calculated from the value for each solvent system.

Table 2. Yield of glycolic acid after evaporation treatment.

Experiment	Glycolate remained [μmol (%)]	
	Free acid[a]	Sodium salt[a]
Exp I	2.00 (50.0)	4.05 (101)
Exp II	2.51 (62.8)	3.93 (98.3)

(Exp I) A solution containing 4 μg of glycolic acid in 40 μl H_2O was added to 4 ml of diethyl ether and evaporated at 45°C for 10 min.

(Exp II) A solution containing 4 μg of glycolic acid in 400 μl H_2O was added to 4 ml of diethyl ether and evaporated at 45°C for 30 min.

After drying, 5 ml of water were added to the solutions and 0.1 ml of each solution was offered to glycolic acid analysis. Each experiment was carried out in duplicate. The same experiments were done on the solution not containing glycolic acid, and the value was subtracted from the above determinants as a background value.

[a] Form of glycolic acid in the original solution.

4. Volatility of free glycolic acid. To examine the loss of glycolic acid during the evaporation of diethyl ether, the amount of glycolate residue remaining was measured after the following two kinds of treatments (I and II), using free acid and sodium salt (Table 2). Four micromoles of glycolate in 40 μl water were mixed with 4 ml of diethyl ether in a 25 ml egg-shaped flask and evaporated to dryness by a tap-water aspiration system for 10 min at 45°C in a water bath. The dried residue was dissolved in 5.0 ml of distilled water, and an aliquot (0.1 ml) was offered to the DON treatment. The result showed that the glycolate as a sodium salt remained completely after the procedure, but glycolate as a free acid lost about half the acid during the evaporation process (Experiment I). In Experiment II, a more diluted sample, 4 μmol of glycolate in 400 μl water, was mixed with 4 ml of diethyl ether, evaporated to dryness for 30 min at 45°C, and the remaining glycolate similarly measured. In contrast to the almost complete recovery of glycolate sodium salt, about

40% of free glycolic acid was lost in the procedure (Experiment II).

5. Efficiency of glycolic acid extraction by diethyl ether under several concentrations of acid. Bacterial cells are generally hydrolyzed by hydrochloric acid. The effect of the concentration of hydrochloric acid used for hydrolysis to extract glycolic acid with diethyl ether was examined. Figure 3 indicates that the glycolic acid extraction was not significantly affected by the presence of 1 to 6 N HCl and 2 N trifluoroacetic acid, though glycolic acid was more effectively extracted under hydrochloric acid of less than 1 N.

II. Effects of various biological compounds to the DON reaction and attempts to remove these effects by solvent works

1. Influence of various biological compounds on the color reaction of glycolic acid in DON reaction. Glycolic acid gives a clear red purple color when reacting

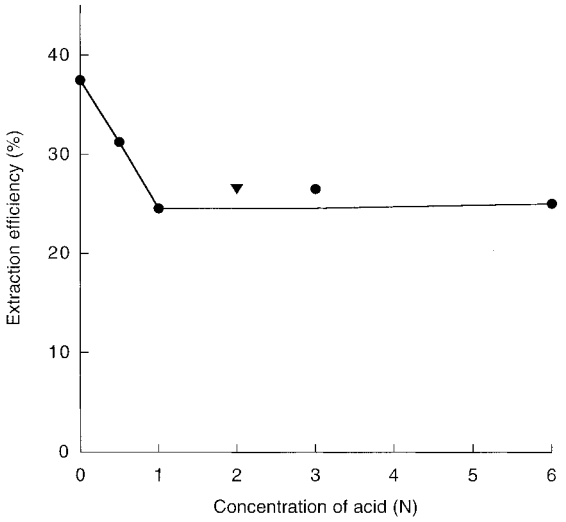

Fig. 3. Efficiency of glycolic acid extraction with diethyl ether under several acid conditions.

The extractions were carried out under conditions of hydrochloric acid (●) or of trifluoroacetic acid (▼).

Table 3. Effects of various compounds on the color reaction yielded by glycolic acid and DON reagent.

Compound	Absorbance at 530 nm	Ratio to blank	Color
Blank (Glycolic acid)	0.313	1.00	Clear red purple
Additive			
(1) Amino acids			
Glycine	0.311	0.99	Clear red purple
Alanine	0.320	1.02	Clear red purple
Valine	0.315	1.00	Clear red purple
Leucine	0.315	1.00	Clear red purple
Isoleucine	0.315	1.00	Clear red purple
Aspartic acid	0.327	1.04	Clear red purple
Glutamic acid	0.308	0.98	Clear red purple
Serine	0.315	1.00	Clear red purple
Threonine	0.317	1.01	Clear red purple
Proline	0.313	0.99	Clear red purple
Cysteine	0.303	0.96	Clear red purple
Methionine	0.136	0.43	Clear red purple
Phenylalanine	0.268	0.85	Clear red purple
Tyrosine	0.303	0.96	Clear red purple
Tryptophan	0.048	0.15	Clear red purple
Arginine	0.313	0.99	Clear red purple
Histidine	0.322	1.02	Clear red purple
Lysine	0.321	1.02	Clear red purple
(2) Sugars and derivatives			
Glucose	n.d.	—	Yellowish brown
Mannose	n.d.	—	Yellow
Galactose	n.d.	—	Yellow
Fructose	n.d.	—	Dark red purple
Arabinose	n.d.	—	Pale blue
Ribose	n.d.	—	Dark red purple
Xylose	n.d.	—	Dark red purple
Mannitol	0.312	0.99	Clear red purple
Sorbitol	0.302	0.96	Clear red purple
Gluconic acid	0.338	1.07	Clear red purple
Glucuronolactone	0.266	0.84	Clear red purple
Glycerol	0.310	0.98	Clear red purple
Furfural	0.292	0.93	Clear red purple
Glucosamine	n.d.	—	Brown
5-oxo-Gluconic acid	n.d.	—	Dark red purple
(3) Carboxylic acids			
Formic acid	0.300	0.95	Clear red purple
Acetic acid	0.317	1.01	Clear red purple
Pyruvic acid	0.289	0.92	Clear red purple
Oxalic acid	0.318	1.01	Clear red purple
Malonic acid	0.316	1.00	Clear red purple
Succinic acid	0.385	1.22	Clear red purple
Fumaric acid	0.316	1.00	Clear red purple
Malic acid	0.224	0.71	Clear red purple
Oxaloacetic acid	0.296	0.94	Clear red purple
Citric acid	0.267	0.85	Clear red purple
2-oxo-Glutaric acid	0.285	0.90	Clear red purple
Levulinic acid	0.339	1.08	Clear red purple
Glyoxylic acid	n.d.	—	Dense violet
Lactic acid	n.d.	—	Opalescent yellow
Tartaric acid	n.d.	—	Dark violet
(4) Fatty acids			
Myristic acid	0.326	1.03	Clear red purple
2-OH-Myristic acid	0.188	0.60	Pale red orange
3-OH-Myristic acid	0.197	0.63	Pale red orange
(5) Bases			
Adenine	0.315	1.00	Clear red purple
Guanine	0.307	0.97	Clear red purple
Cytosine	0.313	0.99	Clear red purple
Thymine	0.318	1.01	Clear red purple
Uracil	0.320	1.02	Clear red purple
(6) Others			
Methanol	0.326	1.03	Clear red purple
Ethanol	0.207	0.66	Clear red purple
Ethyl acetate	0.250	0.79	Clear red purple
Ethyl ether	0.315	1.00	Clear red purple

A sample containing 1 μmol of each of the above compounds and 50 nmol of glycolic acid in 100 μl aqueous solution was treated with 2 ml of DON reagent, diluted with 1.9 ml of 2 N H_2SO_4, and the absorbance was determined at 530 nm.

with DON reagent. Bacterial cell hydrolysis, however, accompanies the liberation of many kinds of biological compounds. Therefore the influences of various biological compounds to the color production in DON reaction were examined. No inhibitory effect was recognized on the color formation of glycolic acid in the excessive presence of amino acids except for methionine or tryptophan, which markedly reduced the intensity of the color yielded by glycolic acid without changing the color character (Table 3). Several sugars and the related compounds gave various intense colors, though the color tones were quite different from that of glycolic acid. Furthermore, strong and characteristic colors arose from coexistent carboxylic acids such as glyoxylic, lactic, and tartaric acids, and a few fatty acids such as 2-hydroxy- and 3-hydroxymyristic acids. These results suggest that cell components will give various interfering effects against glycolic acid detection in DON reaction, and some treatments are required for removing these compounds before the DON reaction of the cell hydrolysates.

2. Separation of the interfering compounds from glycolic acid fraction by diethyl ether extraction. A solution containing one of the possible interfering compounds was extracted by diethyl ether. After being dried, the extracts were subjected to DON reaction (Table 4). Any distinct color was not shown from the extracts of most sugars and amino acids, but those from glyoxylic acid and some other hydroxy acids revealed a weak absorption at 530 nm (column 2). On the other hand, solutions containing both glycolate and one of these compounds were successively extracted with diethyl ether. After the first extracts were discarded, the second and third ones were analyzed as described above. The second extracts from the solutions containing glycolic acid and amino acids or sugars showed normal color formation as the control experiment, but the extracts from the solutions containing glyoxylic acid or some other organic acids still gave some confusing reaction because of its intensity or tone of color (column 3). But these undesirable influences were mostly removed from the third extracts (column 4).

Table 4. Color reactions of extracts by diethyl ether from solutions of various biological materials containing or not containing glycolic acid.

Compound[a]	Absorbance at 530 nm		
	Extract of the compound[b]	Extract of solution containing the compound and glycolic acid[c]	
	1st extract	2nd extract	3rd extract
Fructose	0.012	0.230 (90)	n.d.[f]
Galactose	0.000	0.230 (90)	n.d.
Glucose	0.034	0.256 (100)	n.d.
Mannose	0.012	0.258 (101)	n.d.
Arabinose	0.008	0.238 (93)	n.d.
Ribose	0.004	0.250 (98)	n.d.
Xylose	0.012	0.244 (95)	n.d.
Glucosamine	0.028	0.248 (97)	n.d.
Glycerol	0.024	0.270 (105)	n.d.
Methionine	0.024	0.262 (102)	n.d.
Tryptophane	0.024	0.236 (92)	n.d.
Glyoxylic acid	0.122[d]	0.335 (131)	0.268 (134)
Lactic acid	0.008	0.151 (59)	0.166 (83)
Malic acid	0.022	0.212 (83)	0.188 (94)
Tartaric acid	0.030	0.223 (87)	0.212 (106)
o-Phthalic acid	0.022	0.220 (86)	0.204 (102)
2-OH-Myristic acid	0.082[e]	0.110 (43)	0.182 (91)
3-OH-Myristic acid	0.046[e]	0.084 (33)	0.202 (101)
Blank	—	0.256 (100)	0.200 (100)

[a] The compounds were dissolved in H_2O and acidified with HCl solution.

[b] A 0.2 ml portion containing 1 μmol of each compound was extracted with 2 ml of water-saturated diethyl ether. After being dried up and dissolved in 0.1 ml of H_2O, the solutions were treated with 2 ml of DON reagent, diluted with 1.9 ml of 2 N H_2SO_4 and the absorbance was determined at 530 nm.

[c] The same experiments were done with solutions containing 100 nmol of glycolic acid and 1 μmol of each compound. The first extracts were discarded, and the following 2nd and 3rd extracts were analyzed as mentioned above.

[d] Pale yellow.

[e] Pale orange.

[f] Not determined.

III. Application of the glycolate test with solvent method to bacterial cell hydrolysates and its further improvement

1. Test of solvent systems for glycolic acid extraction from cell hydrolysates of "Micromonospora nigra" JCM 3328. As shown in Table 5, the effect of solvent systems was examined by using cell hydrolysate of a glycolate test positive organism, "*M. nigra*" JCM 3328 (Uchida and Seino, 1997). The first extracts by the solvent systems shown in Table 5 (column 1) showed not the clear reddish color, but some light or dark brown colors by DON reaction (columns 1 and 2). The next extraction with diethyl ether or ethyl acetate (column 3) showed a clear solution of red purple color. The second extraction with ethyl acetate gave more intensive red color than that with diethyl ether (column 4). But the boundary zones between the lysate and ethyl acetate layer were less clear than that of diethyl ether, and it was found difficult to separate the two layers clearly.

2. Absorption spectra of the color appearing in the reactions of extracts of bacterial cell hydrolysates and DON reagent. Figure 4 shows absorption spectra of the reaction colors between DON reagent and each of the first, second, and third extractions with diethyl

Table 5. Comparison of solvent systems for extraction of glycolic acid from cell hydrolysates of "*M. nigra*" JCM 3328.

First extraction		Second extraction	
Solvent	Color of the extract	Solvent	Absorbance at 530 nm after DON reaction
n-Hexane-ether (3 : 1)	Pale yellow	Diethyl ether	0.245
Diethyl ether-ethyl acetate (3 : 1)	Light brown	Diethyl ether	0.599
Diethyl ether	Light brown	Diethyl ether	0.617
Diethyl ether	Light brown	Ethyl acetate	0.951
Ethyl acetate	Dark brown	Ethyl acetate	0.852

Dried cells were hydrolyzed with 6 N HCl at 100°C for 2 h on a block heater. Aliquots (0.2 ml, corresponding to 2.0 mg cells) were shaken with 2 ml each of water-saturated solvent for 1 min in a mixer, centrifuged briefly, and the upper layers were carefully transferred, washed by the same solvent (0.5 ml), and combined (first extraction). After being alkalized with 4 µl of 0.1 N NaOH, the second solvent extraction was carried out. The extracts were completely dried by nitrogen gas streaming. The residue was dissolved in 50 µl H_2O, reacted with 1 ml of DON reagent, and diluted with 0.95 ml of 2 N H_2SO_4. The quantitative determination was done with only the second extracts.

ether from cell hydrolysate of "*M. nigra*" JCM 3328. The spectrum from the first extract showed an unclear peak ranging in a wide absorption area (Fig. 4, line 2). The second and third extracts revealed a strong red purple color, of which the absorption peak was near at 530 nm (Fig. 4, lines 3 and 4). It was almost the same as that of glycolic acid (Fig. 4, line 1). On the contrary, the second diethyl ether extract from *Microbispora rosea* JCM 3006, an organism with an acetyl type cell wall, gave no clear peak by DON reaction (Fig. 5, line 3).

3. Application of the solvent method to cell hydrolysates of various actinomycete strains. The solvent method mentioned above was applied to various actinomycete strains, of which acyl types were already determined by the column method (Uchida and Seino, 1997). Among 20 strains, the results on the acyl type completely coincided with those in the previous paper.

Hydrolysis of bacterial cells was done with each 10 mg of cells in 1 ml of 6 N HCl in a small culture tube at 100°C for 2 h. Each 0.2 ml portion of the hydrolysates (equivalent to 2 mg of bacterial cells) was extracted two times with each 2 ml of diethyl ether (water saturated). The second extracts were alkalized with 5 µl of 1 N NaOH, dried, dissolved with 25 µl of water, and heated with 0.5 ml of DON reagent. The solutions were diluted with 0.5 ml of 2 N H_2SO_4, cooled, and transferred to 1.5 ml plastic tubes. All the tested strains with glycolyl type cell walls showed a clear red purple color by the solvent method (Fig. 6).

4. Miniaturization of the glycolate test. Dried cells of two strains from the genera *Micromonospora* and *Microbispora* were used in the experiment. First, the bacterial cells were hydrolyzed with 6 N HCl at 100°C for 2 h in 1.5 ml plastic tubes equipped with safety locks. From quantitative consideration, this step was carried out with 10 mg of cells and 1 ml of 6 N HCl, though only a small amount of cells could be necessary for the following processes. Next, 20 µl of the cell hydrolysate were taken in a plastic tube equipped with a safety lock and extracted several times with 200 µl each of water-saturated diethyl ether. The second extract was transferred with a slender type of plastic tip into a fresh plastic tube, alkalized with 2 µl of 0.1 N NaOH, and dried up. Third, 50 µl of DON reagent was added to the tube and heated at 100°C for 10 min on a block heater. Figure 7 shows that the small amount of cell lysate equivalent to 0.2 mg of bacterial cells can be used for a determination of acyl type by this method.

Discussion

The solvent method described here has several advantages when it is compared with the column method hitherto used. First, the new method does not need the specially designed glass columns and ion exchange resin such as Dowex 1-acetate type resin (Uchida and Aida, 1977, 1984), both of which are not commercially available, and it also does not essentially require a spectrophotometer. Second, the method requires only a small amount of bacterial cells for the discrimination of the acyl type. For example, bacterial cells of 0.1–1 mg weight are enough to apply to the method. Usually, bacteria of glycolyl type contains about 50–150 nmol of glycolyl residues in 1 mg of dried cells, and only 1 nmol of glycolic acid can be clearly detected with a small amount of DON reagent. Third, all steps of the method can be carried out in simple plastic ware, which is widely used for microscale molecular biological purposes. A polypropylene tube was resistant to heating at 100°C with 6 N hydrochloric acid or with concentrated H_2SO_4, and to repeated shakings with organic solvents such as diethyl ether or ethyl acetate. These results made the solvent method simple and rapid for a great many

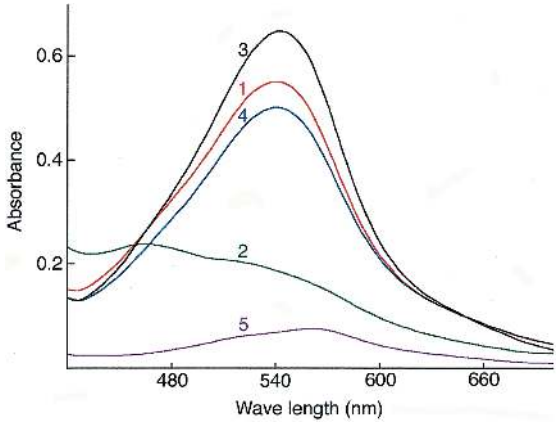

Fig. 4. Absorption spectra of the colors formed by reactions with DON reagent and extracts by diethyl ether from cell hydrolysate of "*Micromonospora nigra*" JCM 3328.

Line 1, glycolic acid; line 2, 1st extract; line 3, 2nd extract; line 4, 3rd extract; and line 5, blank.

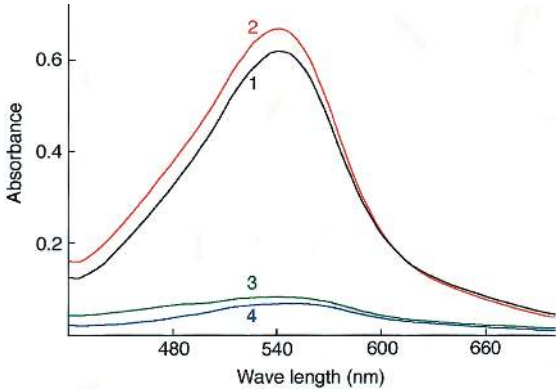

Fig. 5. Comparison of the reaction colors obtained from cell hydrolysates of "*Micromonospora nigra*" JCM 3328 and *Microbispora rosea* JCM 3006.

Line 1, glycolic acid; line 2, 2nd extract from "*M. nigra*" JCM 3328; line 3, 2nd extract from *M. rosea* JCM 3006; and line 4, blank.

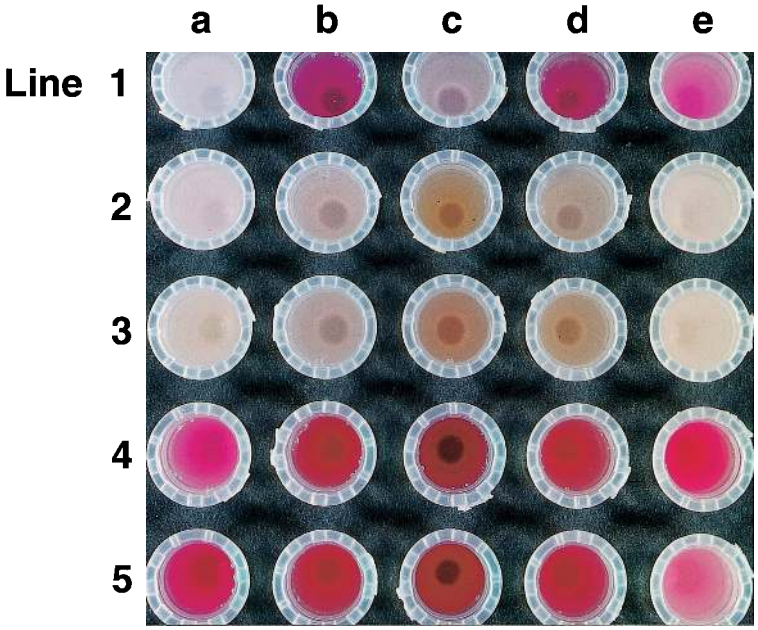

Fig. 6. Color reactions of diethyl ether extracts from cell hydrolysates of various actinomycete strains.

Line 1 (Control experiments): direct colorization on 25 µl H_2O (blank, 1a), 25 nmol of glycolic acid (1b), 2nd extract of 0.2 ml of 6 N HCl (1c), 2nd extract of glycolic acid solution (0.8 µmol/0.2 ml) (1d), and the 3rd extract (1e). The extracts (1c–1e) were dried, dissolved in 160 µl H_2O, and the 25 µl portions analyzed. Cell hydrolysates were examined on the second extract of the following strain (lines 2 to 5). Lines 2 and 3 (acetyl type): *Microbispora rosea* JCM 3006 (2a), *Planobispora longispora* JCM 3092 (2b), *Amycolatopsis mediterranei* JCM 4789 (2c), *Actinomadura madurae* JCM 7436 (2d), and *Nocardiopsis dassonvillei* JCM 7437 (2e) and *Pseudonocardia compacta* JCM 7438 (3a), *Streptosporangium corrugatum* JCM 3181 (3b), *Nocardioides albus* JCM 3185 (3c), *Pseudonocardia hydrocarbonoxydans* JCM 3392 (3d), and *Streptomyces griseus* IAM 12311 (3e). Lines 4 and 5 (glycolyl type): *Mycobacterium phlei* AKU 1574 (4a), *Rhodococcus rhodnii* JCM 3203 (4b), "*Micromonospora nigra*" JCM 3328 (4c), *Nocardia carnea* JCM 3375 (4 d), *Catellatospora ferruginea* JCM 7544 (4e), *Pilimelia anulata* IFO 15533 (5a), *Glycomyces harbinensis* IFO 14487 (5b), *Actinoplanes philippinensis* JCM 3001 (5c), *Gordonia amarae* JCM 3171 (5d), and *Couchioplanes caeruleus* subsp. *azureus* JCM 3246 (5e).

Fig. 7. Miniaturization of the glycolate test with a solvent method.

Bacterial cell hydrolysate (corresponding to 0.2 mg cells) was extracted with 200 µl of diethyl ether. The second extracts were dried up and reacted with 50 µl of DON reagent in capped tubes. 1, blank; 2, 2 nmol glycolic acid; 3, 0.2 mg cell hydrolysate of *Microbispora rosea* JCM 3006 (acetyl type); 4, 0.2 mg cell hydrolysate of "*Micromonospora nigra*" JCM 3328 (glycolyl type). Plastic tubes were used in all steps in the test. Final dilution with 2 N H_2SO_4 was omitted in this experiment.

samples. Furthermore, this method will be applied at a colony level because of the high sensitivity to glycolic acid. For example, it would be used for a selection of some mutants in the biosynthetic pathway of muramyl residues in cell wall peptidoglycan. These mutants will be useful in search of new antibiotics against pathogenic glycolyl types of organisms, such as mycobacteria and nocardias, to mankind, animals, fishes, and plants. From another aspect, the method may be effective in microbial ecology because many bacteria of glycolyl type are thought to play important roles in nature for the decomposing abilities of naturals and harmful artificials.

This paper also describes several other parameters that are basically and practically important for the test: a) effective concentration of DON in the reaction, b) stock conditions of DON reagent, c) volatility of glycolic acid, d) efficiency of extraction of glycolic acid with several organic solvents, e) absorption spectra of reaction colors of glycolic acid and cell extracts after DON reaction, and f) effects of various compounds toward color formation of glycolate. The results from these experiments are helpful for further improvement of the method and for application.

As described above, DON reagent is highly sensitive not only to glycolic acid, but also to many other compounds; thus it is necessary to take care of the quality of reagents, e.g., diethyl ether, H_2SO_4, and DON, and of the contaminants from glasswares, pipettes, tubes, bottles, and others on the leakage from plastic wares.

The miniaturization of the method is also discussed, and the applicability of bacterial cells confirmed at less than 0.2 mg with a small amount of reagent.

Procedure of glycolate test by solvent method. One milligram of actinomycete cells are hydrolyzed with 0.1 ml of 6 N HCl in small glass culture tubes or suitable polyethylene tubes with tight caps at 100°C for 2 h. The cell hydrolysates are extracted with 1 ml of diethyl ether (water saturated). After the first extract is discarded, a second extract by the same manner is transferred into an another tube, alkalized with 2 µl of 0.1 N NaOH, and dried up. The residue is dissolved in 500 µl of DON reagent. The tube is capped and heated at 100°C for 10 min. Bacterial cells of glycolyl type show a clear red color, whereas cells of acetyl type reveal almost no color. Both types of known actinomycete cells should be included as references.

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