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New Medium for Isolating Propionibacteria and Its Application to Assay of Normal Flora of Human Facial Skin

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The conditions for isolation and cultivation of *Propionibacterium acnes* and related propionibacteria were studied in detail. Triton X-100 added to the diluent inhibited the growth of propionibacteria in concentrations of 0.05 to 0.1%. However, such was not the case with Tween 80; rather, growth of the bacteria was further enhanced by this agent. Consequently, Tween 80 was considered to be a suitable surfactant for addition to the diluent for isolation of propionibacteria. A new medium for isolating propionibacteria from human skin was developed. Comparative studies with colonies of *P. acnes*, *Propionibacterium granulosum*, and *Staphylococcus epidermidis* showed morphological differences among the colonies; thus, the medium was very useful for differentiating and identifying species of the microbes. The new medium was used for studies on the distribution of propionibacteria on the foreheads of 30 Japanese volunteers. Among 447 strains of *P. acnes* and 86 strains of *P. granulosum* isolated from the volunteers, all strains of the former were positive for indole, nitrate, milk, and gelatin hydrolysis, whereas all strains of the latter were negative for all of the tests.

Most of the work done previously on the isolation of propionibacteria from human skin was concerned with quantitative studies dealing mainly with the number of bacteria (4, 6, 17). Precise comparisons of the methodology for isolation and cultivation of the microbes are few (11). For example, the diluent for aerobes first described by Williamson and Kligman (26) has been used for isolation of propionibacteria without further examination of the suitability of the composition and of the conditions used. Although the problems pertaining to the taxonomy of *Propionibacterium acnes* were largely dealt with by Johnson and Cummins in 1972 (2, 8, 13-15, 18, 20), it is apparent in the literature that there are circumstances in which *Propionibacterium granulosum* are often confused with *P. acnes* (1, 21, 24, 25).

We report herein a new diluent and medium for accurate isolation of propionibacteria and discuss the conditions suitable for the tests used in classifying the bacteria. The results of practical application in assays of normal skin flora from Japanese volunteers are also included.

MATERIALS AND METHODS

Bacteria and media. *P. acnes* ATCC 11827, 10^3 cells per ml, was suspended in two different diluents to assess the effects of the diluents on preservation of the bacteria. The diluent was 1 mM phosphate buffer supplemented with 0.1% Triton X-100 or 1 mM phosphate buffer supplemented with 0.1% Tween 80. The suspensions were stored at 0, 25, and 37°C. After 1, 2, 4, and 17 h, 0.1 ml of the bacterial suspension was smeared onto two plates composed of: Trypticase (BBL Microbiology Systems), 1.5%; yeast extract (BBL), 0.5%; heart extract (Nissui), 0.5%; glucose, 0.3%; NaCl, 0.2%; K_2HPO_4, 0.2%; L-cysteine HCl, 0.03%; agar, 1.5%; Tween 80, 0.025% (pH 6.8). The number of viable cells in each diluent was counted after 4 days of anaerobic incubation (CO_2-N_2, 10%: 90%) at 37°C. For inhibition tests, five strains of propionibacteria were inoculated into tubes of test medium (composition similar to that described above, except that the agar concentration was reduced to 0.1%) which contained different concentrations of Triton X-100 or Tween 80. The growth of propionibacteria was assayed by measurement of the optical density at 660 nm in a spectrophotometer.

Method of isolation. For isolation of propionibacteria from human skin, the medium given in Table 1 was used. A 1-cm^2 area of the forehead of a volunteer was cleansed with a sterile swab, and the swab was placed in a test tube containing 10 ml of diluent (Table 1). Tenfold dilutions were made to count the number of bacteria, and 0.1 ml of each dilution was inoculated on the plates. The test tubes were kept in an ice bath during the entire procedure. After 7 days of anaerobic incubation at 37°C, colonies with different morphology (yellow, slightly yellow, and brownish) appeared in numbers of approximately 20 to 100 per plate. They were counted and classified.

Biological and biochemical tests. The characteristics of 533 strains of anaerobes isolated from human skin were tested by the following methods. The composition of the test medium used was as follows (in percent, wt/vol): Trypticase (BBL), 1.0; yeast extract (BBL), 0.3; heart extract (Nissui), 0.3; NaCl, 0.2; L-cysteine HCl, 0.03; agar, 0.1; Tween 80, 0.025 (pH

TABLE 1. *Composition of new medium and diluent*

Ingredient	Amt (%)
Medium (pH 6.8)	
Trypticase (BBL)	1.5
Yeast extract (BBL)	0.5
Heart extract (Nissui)	0.5
Glycerol	1.0
NaCl	0.2
K_2HPO_4	0.2
L-Cysteine HCl	0.03
Sodium oleinate	0.005
Bromocresol purple	0.002
Agar	1.5
Diluent (pH 6.8)	
KH_2PO_4	0.5
Na_2HPO_4	0.4
Tween 80	0.1
L-Cysteine HCl	0.03

7.0) (9). Gram-positive, non-sporeforming anaerobic rods were classified following the guidelines of the *Anaerobe Laboratory Manual* (7). The production of volatile fatty acids was analyzed by gas chromatography. Catalase, esculin hydrolysis, indole, and nitrate reduction were examined with 3% H_2O_2, 1% ferric ammonium citrate solution, Kovacs reagent, and nitrate reagents (0.33% sulfanilic acid and 0.5% α-naphthylamine in 30% acetic acid), respectively. Gelatin hydrolysis was examined by comparison with findings in the uninoculated control tube after storage in ice water for over 30 min. This test and the curd formation, as well as digestion of milk, were carried out daily for 21 days. Sugar fermentation tests as examined by pH meter were as described elsewhere (9). All test media were inoculated by using Pasteur pipettes and incubated at 37°C. The antibacterial susceptibility, i.e., the minimal inhibitory concentration, of 11 different antibiotics for 70 strains among 533 isolates was determined by the standard agar dilution technique. Serotyping of *P. acnes* was carried out by the agglutination test (9).

RESULTS

Effects of Triton X-100 and Tween 80 on preservation of *P. acnes*. Triton X-100 was bactericidal at all temperatures tested (Table 2). After 1 h, the count of *P. acnes* decreased from 1/2 to 1/20 of the initial count, and at 25 and 37°C there was no recovery after 4 and 17 h, respectively. However, such was not the case with Tween 80, except at 37°C. After 4 h, the count of viable cells was much the same as the initial count at 0 and 25°C, and at 0°C the cell count was constant up to 17 h.

Effects of Triton X-100 and Tween 80 on inhibition of growth of propionibacteria. Triton X-100 partly inhibited the growth of all species tested except *P. lymphophilum* at a concentration of 0.01% (Fig. 1). *P. acnes*, *P. granulosum*, and *P. lymphophilum* were markedly inhibited at 0.05 to 0.1%. Tween 80 did not exert an inhibitory effect on any preparation at a concentration of up to 2%. Consequently, Tween 80 as the surfactant in the diluent was considered to be more suitable than Triton X-100.

TABLE 2. *Effects of Triton X-100 and Tween 80 on preservation of P. acnes ATCC 11827*

Surfactant[a]	Storage temp (°C)	No. of colonies per plate after preservation time in air of:				
		0 h	1 h	2 h	4 h	17 h
Tween 80	37	2,730	2,480	872	13	0
	25	2,520	2,610	2,330	2,290	20
	0[b]	2,580	2,530	2,460	2,470	2,570
Triton X-100	37	2,590	104	11	0	0
	25	2,680	1,690	291	55	1
	0	2,580	1,760	1,140	576	121

[a] The concentration of surfactants was 0.1% in 1 mM phosphate buffer.
[b] In ice water.

Isolation of propionibacteria with the new medium. Among the colonies recovered on the plates after 7 days of anaerobic incubation at 37°C, we found four distinct colony types (Fig. 2). With regard to the results of classification of 533 isolates, the colony types on our new medium plates differed among the species; i.e., type 1 was *P. granulosum*, types 2 and 3 were *P. acnes*, and type 4 was *Staphylococcus epidermidis* (Table 3).

Biochemical characteristics of the isolates. The reactions of 447 isolates of *P. acnes* and 86 isolates of *P. granulosum* are summarized in Table 4. Indole, nitrate, milk, and gelatin hydrolysis were positive in all isolates of *P. acnes*, but negative in all *P. granulosum* isolates.

Susceptibility of isolates to antibacterial agents. Seventy isolates of *P. acnes* and *P. granulosum* were resistant to aminoglycosides, except to midecamycin. *P. acnes* was more sensitive to cephaloridine than was *P. granulosum*. There were no differences in the effects of 11 antibiotics between two serotypes of *P. acnes* (Table 5).

Population of propionibacteria on the forehead. The distribution of propionibacteria on the foreheads of 30 volunteers is summarized in Fig. 3. The number of bacteria per 1 cm^2 ranged from 10^2 to $>10^6$. The mean number per 1 cm^2 was slightly higher in men (10^5) than in women (10^4). Almost no variation was found in the number of propionibacteria in individual subjects upon repeated testing (Fig. 4). The species of propionibacteria isolated from the forehead were *P. acnes* and *P. granulosum* (ratio, between 10:1 and 100:1).

DISCUSSION

In 1965, Williamson and Kligman reported a method for isolating human skin flora (26). Since then most investigators have used their pro-

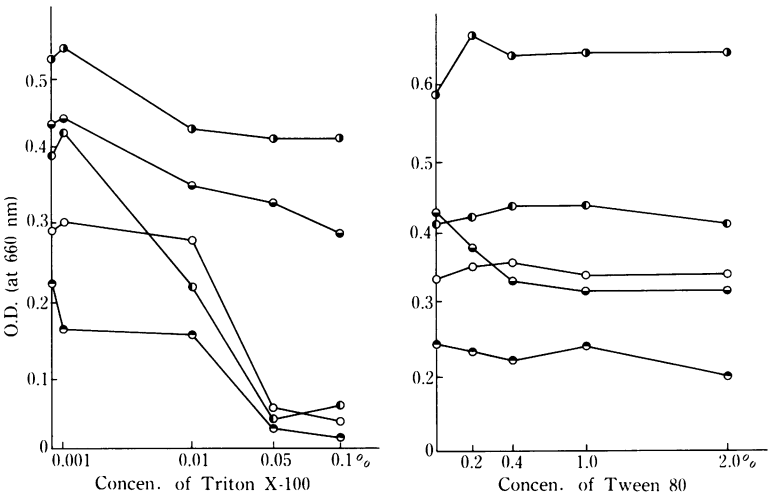


FIG. 1. *Effects of Triton X-100 and Tween 80 on growth of propionibacteria after incubation for 40 h at 37°C. Symbols: ◑, P. avidum ATCC 25577; ◒, P. acidi-propionici 1052 (isolated from feces); ◐, P. granulosum ATCC 25564; ○, P. lymphophilum 1041 (isolated from dental material); ◓, P. acnes ATCC 11827. O.D., Optical density.*

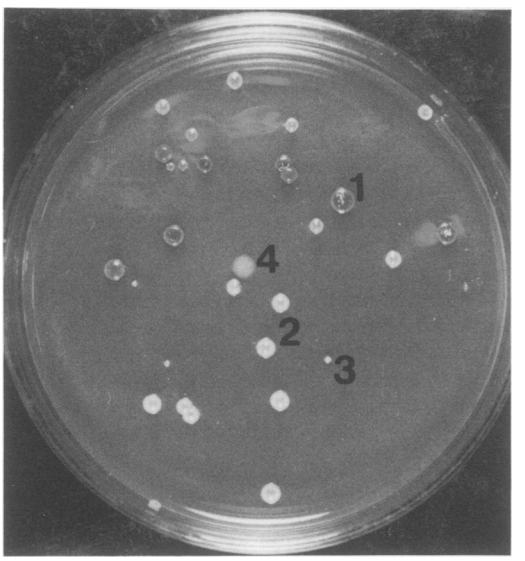


FIG. 2. *Colonial morphology of propionibacteria. The specimen shown was from the nose of a Japanese volunteer. The new medium was used, and the plate was photographed after 7 days of incubation at 37°C. (Specimens from the forehead show a similar pattern.) See Table 3 for explanation of colony types.*

TABLE 3. *Relation between colony type and species of propionibacteria*

Colony type	Color	Size (mm)	Species
1	Brownish (opaque)[a]	2–3	*P. granulosum*
2	Yellow or slightly yellow (opaque)	1.5–2.5	*P. acnes*
3	Yellow or slightly yellow (opaque)	0.5–1	*P. acnes*
4	Whitish (translucent)	0.5–2	*S. epidermidis*

[a] Observed by transmitted light.

TABLE 4. *Characteristics of P. acnes and P. granulosum from human skin*

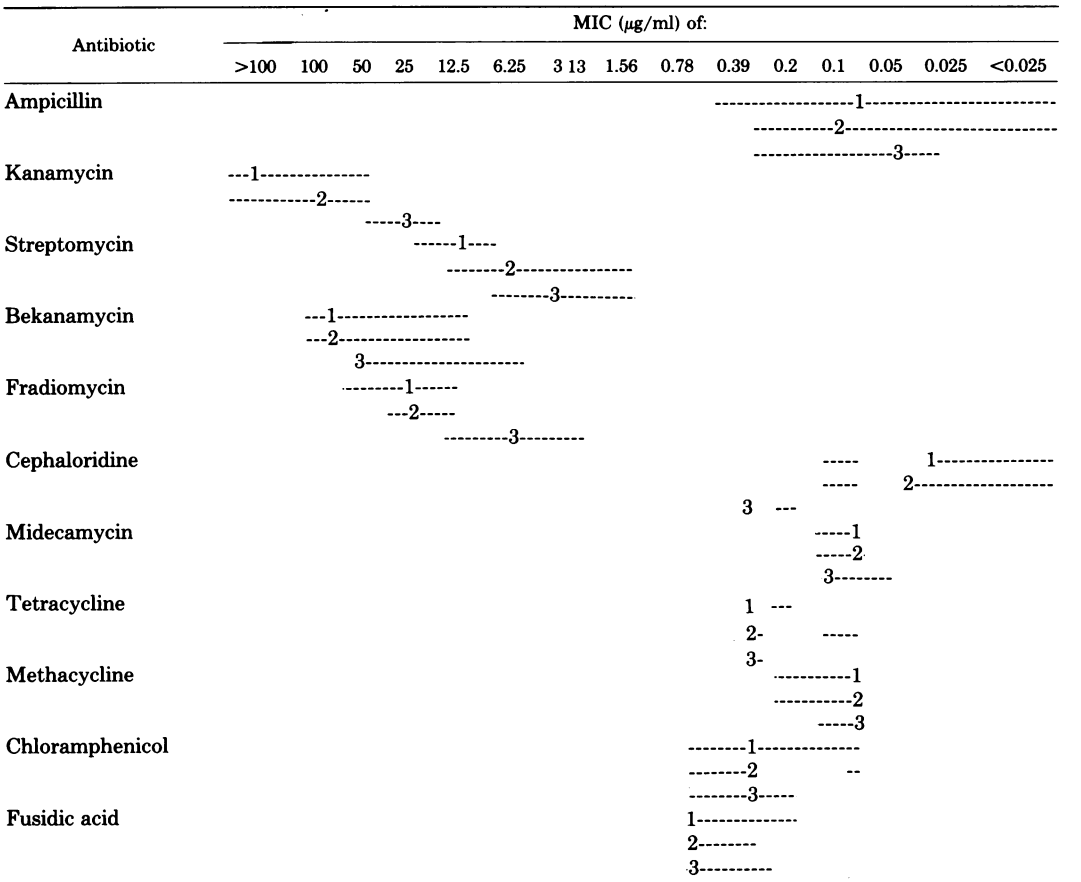
Characteristic	*P. acnes* (n = 447)	*P. granulosum* (n = 86)
Volatile fatty acids (C_2, C_3, and iC_5)[a]	+[b]	+
Esculin hydrolysis	−	−
Catalase	+	+
Indole	+	−
Nitrate	+	−
Gelatin hydrolysis	+	−
Milk	+	−

[a] Acetic, propionic, and isovaleric acids.
[b] + = 100% positive; − = 100% negative.

posed diluent (26) for isolating propionibacteria (3, 12, 19). On the other hand, Noble and Somerville observed that Triton X-100 had a bactericidal effect on the growth of diphtheroides, particularly lipophilic diphtheroides (16).

We found that Triton X-100 inhibited the growth of propionibacteria but that Tween 80 did not. Williamson and Kligman's diluent may be suitable for aerobic organisms (26), but not for propionibacteria. However, such was not the case with Tween 80, and according to our data, the growth of propionibacteria was further stim-

TABLE 5. *Distribution of minimal inhibitory concentrations (MIC) against P. acnes and P. granulosum isolated from normal human skin*[a]



[a] --1--, *P. acnes* serotype I (36 strains); --2--, *P. acnes* serotype II (10 strains); --3--, *P. granulosum* (24 strains). The positions of 1, 2, and 3 indicate the mean value of distribution, and the dashed lines indicate the MIC range.

ulated by 0.005 to 0.01% Tween 80 (data not shown). Consequently, Tween 80 was considered to be an effective surfactant of the diluent for isolation of propionibacteria. For preservation of the anaerobic population, as shown by our data on propionibacteria, it probably is also important to store the tube containing our new diluent in ice water during the entire isolation process.

The new medium was formulated on the basis of a study on optimum conditions for isolating propionibacteria. The characteristics of this medium are as follows. (i) The pH of this medium was adjusted to the optimum for growth of propionibacteria, and growth was inhibited when the pH exceeded 7.0. (ii) A solution of sodium oleinate (final concentration, 0.005%) was sterilized by filtration and was added to other autoclaved components to stimulate the growth of propionibacteria (data not shown) and also to inhibit the growth of *Arachinia* and *Actinomyces* strains (22). (iii) Most propionibacteria fermented glycerol, and the pH of the cultures was in the range of 4.5 to 5.0. Therefore, the colonies of propionibacteria and the surface zones around them on the plates turned yellowish, but such was not the case with *S. epidermidis*.

In this work, biochemical tests on 533 isolates were performed as a check on the accuracy of the classification by colony types; 100% agreement was obtained. The colonial morphology of *P. acnes* and *P. granulosum* on our plates was much the same as that seen on CYLG agar, as reported by Marples et al. (11). Our new medium was not effective for other human materials, for example, dental specimens and feces, but the selective medium for propionibacteria was prepared by adding three compounds to our basic medium formula (22). The supplements were as follows: sodium azide, 0.002%; gentamicin sul-

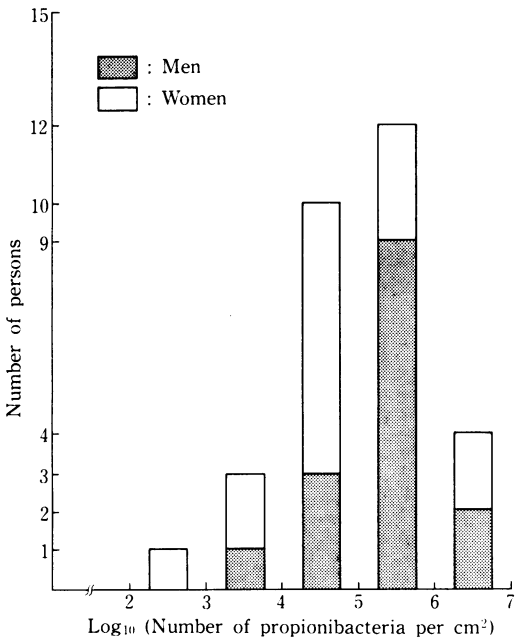


FIG. 3. *Number of propionibacteria per 1 cm² of forehead (n = 30).*

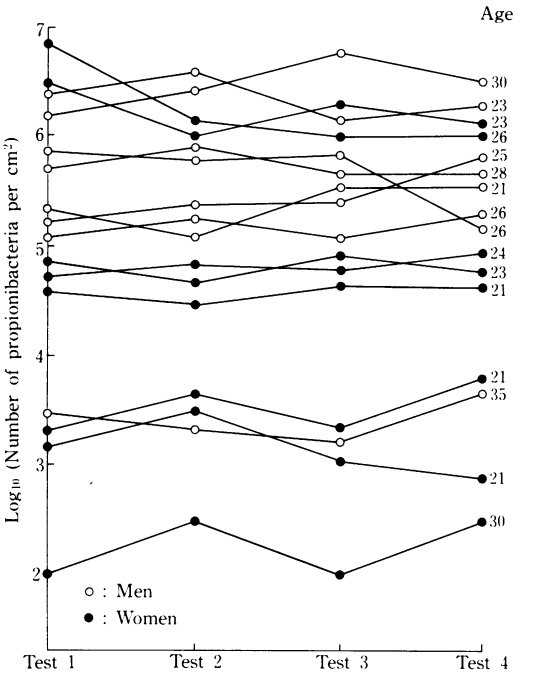


FIG. 4. *Number of propionibacteria on the forehead in repeated tests (n = 16).*

fate, 0.0002%; and Panfran-S {3-di(hydroxymethyl)amino-6[(5-nitro-2-furyl)vinyl]-1,2,4-triazine; Toyama Kagaku, Tokyo, Japan}, 0.00005%.

Indole, nitrate, milk, and gelatin hydrolysis-negative *P. acnes* isolates have been reported by other investigators (7, 10, 12, 21), but in our present work, these reactions were positive on all isolates of *P. acnes*. On the other hand, these reactions were negative on all isolates of *P. granulosum*. Thus, these characteristics should enable a rapid differentiation of the two species. SIM medium, indole-nitrate medium, and thioglycolate medium were not suitable for the indole test, and glucose must be omitted from this test medium. Thiogel medium was not effective for gelatin hydrolysis. In addition, the preparations should be examined after the cultures have been stored in ice water for over 30 min. By our methods, indole and nitrate were positive within 4 days, and milk and gelatin hydrolysis were positive within 7 days, on all isolates of *P. acnes*. Isolates of *P. granulosum* fermented maltose but did not ferment adonitol and erythritol. Ribose fermentation varied with the individual isolates. We have already biotyped *P. acnes* and *P. granulosum* by using carbohydrate fermentation (9, 23).

In agreement with the findings of Evans (5), we conclude that the number of propionibacteria on the forehead was in the range of $<10^2$ to 10^6 per cm², and these patterns are probably constant among individual subjects.

Our results are also in concert with those of McGinley and co-workers (12); i.e., the isolation ratio of *P. acnes* and *P. granulosum* was between 10:1 and 100:1. *P. avidum* could not be isolated from the facial skin of Japanese volunteers.

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