Nitrogen Requirements and Uricolytic Activity of Cutaneous Bacteria

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Uric acid, but not xanthine, was degraded by gram-positive catalase-producing cocci and diphtheroids which represented the two predominant human autochthonous skin bacteria. The proportions of uricolytic cocci and diphtheroids varied with the cutaneous site sampled. Uric acid and allantoin were not utilized by cocci or diphtheroids as sole sources of nitrogen. Uric acid appeared to act only as a secondary substrate for the gram-positive bacteria. Cutaneous cocci are known to be ureolytic but few diphtheroids had urease activity. Urea and ammonium nitrogen were not utilized as sole nitrogen sources by cocci, but some diphtheroids used these compounds for nitrogen. The majority of the cocci and diphtheroids were nutritionally fastidious and required amino-nitrogen for growth. In addition, some strains required vitamins and other unidentified metabolites found in yeast extract. These requirements were partially related to the cutaneous site from which the cocci or diphtheroids were isolated. Certain gram-negative bacilli degraded uric acid and utilized urate or its degradation products as nitrogen sources.

The human integument and its microbial flora comprise a specific ecosystem. In attempting to characterize intrinsic factors that regulate any ecosystem, representative organisms of a habitat must be tested for their ability to utilize all known primary and intermediate substrates suspected in the ecosystem (9). Amino acids, ammonia, urea, uric acid, and creatinine are the most common skin products that may act as nitrogen compounds for autochthonous skin organisms (11). Few studies have dealt with determining which nitrogenous compounds produced by skin are utilized or degraded by cutaneous bacteria. Certain aspects of such an investigation and its implications in the nutrition of skin bacteria are reported here.

MATERIALS AND METHODS

Organisms. Gram-positive catalase-producing cocci were isolated on mannitol salt agar (BBL) and separated into staphylococci or micrococci by the method of Baird-Parker (2). Gram-positive diphtheroids or bacilli were isolated and partly differentiated on Furoxone-Tween 80-Oil Red O (FTO) agar (20). Gram-negative bacilli were isolated on Herellea agar (Difco). Yeasts were identified by Gram stain. The sources of reference strains and the maintenance of all cultures were previously reported (19). Undesignated named species were departmental strains.

Swabbing and enumeration techniques. Cotton swabs were moistened in triton-phosphate buffer (24) and rubbed firmly 12 times over various skin sites not greater than 9 cm² in area. Fifteen normal adults were used for sampling. Each swab was placed in 5 ml of buffer and mixed mechanically on a tube vibrator for 30 sec. The swab was discarded and serial dilutions were made. Samples (0.1 ml) of dilutions were spread on plates with glass rods. For elective enrichment, 0.1 ml of a 1-to-50 dilution of the swab rinse was added to a series of minimal salts glucose broths (17) containing 0.15% concentrations of different nitrogen sources. These included NH₄Cl or NH₄NO₃, allantoin, uric acid, and urea. The last two compounds were filter sterilized. Each broth was supplemented with 0.1% Tween 80 (Atlas Powder Co., Wilmington, Del.) to support growth of lipophilic diphtheroids (19). Brain Heart Infusion broth (BBL) with 0.1% Tween 80 was inoculated with each series of minimal broths as a growth control. Skin samples that did not produce growth in the control were not included in any of the data presented in the results. The controls and minimal media were examined daily for evidence of growth or were held 7 days before discarding as negative. When growth occurred, 0.1 ml of broth was transferred to the same respective medium and this was continued for three consecutive transfers. Identification of bacteria from each broth was made by Gram stain and by streaking mannitol salt, FTO, or Herellea media.

Other test media. Degradation of uric acid and xanthine (Nutritional Biochemical Corp., Cleveland, Ohio) was determined by using the method of Gordon and Mihm (8). A 0.8-g quantity of each purine was added to 200 ml of nutrient agar (Difco) and was autoclaved at 121 C for 15 min. In other experiments,
to avoid heating the substrates, 0.8 g of uric acid or xanthine was suspended in 70% ethyl alcohol overnight to render them free of contamination. The alcohol was decanted and sterile, melted nutrient agar was added to the flasks at 47 C. Other media used included ammonium phosphate agar (BBL) and Christensen urea agar (Difco). Pure cultures from 48-hr nutrient slants or broth were used to inoculate these media. Before inoculating minimal media with specific nitrogen substrates, cells were washed in phosphate buffer adjusted to an optical density of 0.5 at 650 nm with a Bausch & Lomb Spectronic-20 colorimeter. A 0.05-ml quantity of washed cells was used to inoculate minimal media. All media and tests were incubated aerobically at 35 C. In some experiments, ammonium phosphate agar was supplemented with yeast extract (Difco), vitamin-free Casitone (Difco), and various vitamins. The vitamins were sterilized by filtration. Blood agar base (Difco) with 0.1% Tween 80 was used with these media as a growth control.

RESULTS

A total of 94 cutaneous bacteria and 15 reference strains were examined for their ability to degrade xanthine and uric acid (Table 1, Fig. 1). Brevibacterium linens was the only organism tested that degraded xanthine and uric acid. Uricolytic action by cutaneous and other bacteria was found. Of the cutaneous strains examined, the lipophilic diphtheroids were most frequently uricolytic. B. linens and Enterobacter aerogenes grew in minimal salts glucose medium with uric acid as a sole nitrogen source, but none of the cutaneous isolates had this ability.

Autoclaving rendered uric acid more susceptible to degradation, but 75% of the strains that degraded autoclaved uric acid also degraded unheated substrate. Autoclaved uric acid medium was used in the remaining experiments. Uric acid medium was held at 35 C for 5 days before being discarded, but many strains produced complete clearing of the uric acid sediment around and under the colonies in 3 days.

Swabbings from five cutaneous sites revealed variations in total aerobic bacterial counts, but each sample contained some uricolytic bacteria (Table 2). Gram stains were made of uricolytic colonies taken from high dilution plates of each sample. Gram-positive cocci were observed most frequently in the nasal, sternum, and toeweb areas, but the groin harbored a predominately uricolytic diphtheroid flora. The axillary site contained an abundance of uricolytic gram-negative bacilli. In elective enrichment media containing specific nitrogen sources, only the axillary sample contained bacteria capable of utilizing uric acid, allantoin, urea, or NH₄Cl. Isolates from these broths were all gram-negative bacilli. The same five skin sites were examined again by the plating-broth procedure on two different occasions. The results were similar to the first experiment. The groin contained primarily a uricolytic diphtheroid population. Gram-positive cocci were the major uric acid-degrading bacteria in the other samples. Gram-negative bacilli were not encountered in the two additional experiments, and there was no corresponding growth in any of the minimal salts media.

Screening of skin sites was continued employing elective enrichment to identify cutaneous bacteria, using urea or ammonia as sole nitrogen sources. Twenty samples from the scalp, face, nasal, groin, and toeweb areas were examined for the presence of urea-utilizing organisms. Two yeast strains, two diphtheroids, and one sporeforming bacillus that utilized urea as a sole source of nitrogen were found in five of the twenty samples. No gram-positive cocci were isolated. A group of 224 lipophilic diphtheroids randomly isolated from various skin sites on FTO agar was tested for urea hydrolysis. None was positive after incubation for 7 days. Of 34 additional skin samples taken, 21 contained ammonium-utilizing bacteria (Table 3). Gram-negative bacilli were isolated from all 21 positive broths, but only 5 of the samples contained ammonium-utilizing diphtheroids. Gram-positive cocci were not recovered. A group of 20

### Table 1. Degradation of uric acid by cutaneous and other bacteria

| Organism                  | No. positive/No. tested |
|---------------------------|-------------------------|
| Cutaneous isolates        |                         |
| Staphylococci             | 8/24                    |
| Diphtheroids (lipophilic) | 46/60                   |
| Gram-negative bacilli     | 4/10                    |
| Reference strains         |                         |
| Staphylococcus aureus     | 4/4                     |
| Corynebacterium xerosis   | 1/1                     |
| ATCC 373                  |                         |
| C. minutissimum           | 1/1                     |
| ATCC 23347                |                         |
| C. striatum               | 1/1                     |
| ATCC 6940                 |                         |
| C. diphtheriae            | 1/1                     |
| C. bouis ATCC 7715 and NIRD 65, 66 | 1/3                  |
| Brevibacterium linens ATCC 9172 | 1/1                |
| Proteus vulgaris          | 1/1                     |
| Escherichia coli          | 1/1                     |
| Enterobacter aerogenes ATCC 8308 | 1/1              |

* Plates were incubated for 5 days. Only those strains producing complete clearing of pure crystals around and under colonies were scored as positive. Abbreviations: ATCC, American Type Culture Collection; NIRD, National Institute for Research in Dairying, Reading, England.

b Strain 66 was positive.
FIG. 1. Two uricolytic Staphylococcus aureus strains and two S. epidermidis-negative strains. Nutrient agar containing 0.4\% uric acid; 5 days incubation.

| Site       | No. of bacteria per swabbing$^a$ | Growth in minimal broth-nitrogen source | Identification of uricolytic colonies |
|------------|---------------------------------|----------------------------------------|--------------------------------------|
|            | Total                           | Uricolytic | Uric acid | Allantoin | Urea | NH$_4$Cl | Gram-positive | Gram-negative rods |
| Nasal      | $6 \times 10^7$                 | $2.5 \times 10^4$ | -         | -         | -     | -        | 22           | 0 | 0 |
| Axilla     | $6.25 \times 10^8$             | $-^b$       | +         | +         | +     | +        | 10           | 4 | $-^b$ |
| Sternal    | $3.25 \times 10^8$             | $2.5 \times 10^4$ | -         | -         | -     | -        | 7            | 0 | 0 |
| Groin      | $4 \times 10^8$                | $2.3 \times 10^7$ | -         | -         | -     | -        | 2            | 26 | 0 |
| Toeweb     | $5 \times 10^7$                | $2.0 \times 10^7$ | -         | -         | -     | -        | 22           | 2 | 0 |

$^a$ Total counts were made on nutrient agar containing Tween 80 and uric acid. Uricolytic colonies were then counted separately.

$^b$ Plates contained confluent areas of uricolysis caused by gram-negative rods.

staphylococci and 4 micrococci isolated from skin were tested for their ability to initiate growth on ammonium phosphate agar. None of these strains grew unless the medium was supplemented with 0.05 to 0.1\% yeast extract.

Since yeast extract is a source of many growth factors, experiments were conducted to determine the relative importance that amino-nitrogen and certain vitamins might have in the nutrition of cutaneous bacteria. A group of 18 cocci and 19
diphtheroids were inoculated on ammonium phosphate agar containing various supplements (Table 4). Of 37 strains tested, 15 required amino-nitrogen for growth, whereas 4 other strains required at least 4 vitamins in addition to amino-nitrogen. Seventeen other strains were more fastidious and grew only on ammonium phosphate medium containing yeast extract. The collective addition of thiamine, riboflavin, nicotinic acid, pantothenic acid, choline chloride, folic acid, nicotinamide, and pyridoxal hydrochloride in amounts of 1 μg/ml to the basal ammonium phosphate agar did not support the growth of any of the 36 strains that grew on media containing Casitone or yeast extract.

The strains examined appeared to be equally fastidious, but the group tested had been previously isolated at random from numerous skin sites. A more direct assessment of the amino acid-vitamin requirements of cutaneous cocci and diphtheroids was made by enumeration of nasal, axillary, groin, and toeweb areas (Table 5). Bacteria capable of growing on basal ammonium phosphate agar were found only in the axilla. These organisms were all diphtheroids. The majority of the bacteria from the axilla, nasal, and toeweb areas were fastidious and required peptone-containing media or amino acids and other metabolites found in yeast extract. The bacterial flora of the groin, which was dominated by diphtheroids, did not contain ammonium-utilizing organisms but the population in general had no requirements for media containing vitamins. This experiment was repeated on two more occasions with similar results. Since the axillary flora was consistently low, however, ammonium-utilizing bacteria were not always detected. Gram-negative bacilli were not found in the samples taken.

Table 4. Role of amino-nitrogen and certain vitamins in the nutrition of cutaneous cocci and diphtheroids

| Organism | No. tested | Basal medium | No. of strains growing on ammonium phosphate agar with various supplements | 1% Vitamin-free (V/F) Casitone | Vitamin-free Casitone + vitamins | 0.25% Yeast extract |
|----------|------------|--------------|-------------------------------------------------------------------------|-------------------------------|---------------------------------|-------------------|
| Cocci .  | 18         | 0            | 0                                                                       | 6                             | 2                               | 10                |
| Diphtheroids . | 19 | 0            | 1                                                                       | 9                             | 2                               | 7                 |

* Vitamins (μg/ml): thiamine, 0.1; riboflavin, 0.2; nicotinic acid, 3.0; pantothenic acid, 0.1. All media contained 0.1% Tween 80. Plates were incubated aerobically for 7 days.

Table 5. Variations in the nutritional requirements of gram-positive cutaneous bacteria by site examined

| Site examined | Estimated no. | Ammonium phosphate agar | 0.25% Yeast extract |
|---------------|---------------|--------------------------|-------------------|
|               | Total         | Cocci                    | Diphtheroids      | Basal | 1% Vitamin-free Casitone | 1% Vitamin-free Casitone + vitamins |                  |
| Nasal         | 435,000       | 400,000                  | 35,000            | <50   | 42,500                     | 62,500                      | 445,000          |
| Axilla        | 5,000         | 1,350                    | 3,650             | 400   | 2,000                      | 1,650                       | 5,000             |
| Groin         | 42,000,000    | 1,000,000                | 41,000,000        | <50   | 41,500,000                 | 42,500,000                 | 43,500,000       |
| Toeweb        | 35,000,000    | 7,000,000                | 28,000,000        | <50   | 4,300,000                  | 5,500,000                  | 42,000,000       |

* All plates were incubated aerobically for 6 days. Total counts were made on blood agar base with 0.1% Tween 80. The approximate number of cocci and diphtheroids in each sample was estimated by counting each group on mannitol salt agar and FTO agar and comparing these counts to the total count. Fastidious bacteria were considered as those strains in each population that grew on total count medium or on the ammonium phosphate agar with yeast extract added. Theoretically, the counts on these two media should have been equal. The vitamins were the four previously used (Table 4).
DISCUSSION

Uricolytic activity was found in the two major human autochthonous skin bacteria, the gram-positive catalase-producing cocci and the diphtheroids. Certain corynebacteria have been reported that degrade uric acid (10). Neither the cocci nor diphtheroids from skin appeared to utilize uric acid as a sole nitrogen source, indicating a need for more complex nitrogen compounds or other unknown growth factors. This was evident from the failure of elective enrichment techniques to recover from the preponderance of cocci and diphtheroids on skin any of these bacteria growing in uric acid broth. Uricolytic action by the gram-positive flora may be an incomplete process. There is evidence that only those organisms that use uric acid as a sole nitrogen source produce all of the enzymes necessary to degrade purines to ammonia (1). Nocardia species were found that degraded hypoxanthine or xanthine but not always both compounds (9). Nocardia species do not contain allantoinase, an enzyme that is normally required for purine degradation, yet they produce urease and utilize urea or ammonia for nitrogen (23). Action on uric acid by the cutaneous flora as a secondary substrate may provide a means by which uric acid is partially metabolized or disposed of on skin. A similar role for facultative uricolytic intestinal bacteria has been proposed (3).

Utilization of ammonium phosphate by micrococci but not staphylococci has been used to separate these two generic groups. Both groups occur regularly on human skin (15) but none of the cutaneous samples examined contained ammonium-utilizing cocci. Baird-Parker (2) found only 77 of 677 micrococci that grew on ammonium phosphate agar, and 42 of these strains grew poorly. The ammonium utilization test does not appear to be a reliable means of separating micrococci from staphylococci.

The addition of yeast extract to ammonium phosphate agar supported the growth of cutaneous cocci and diphtheroids. The basic constituent in yeast extract required for growth appeared to be amino-nitrogen, although vitamins and unidentified metabolites in yeast extract were required to support the maximum number of cocci and diphtheroids in enumeration studies. The relatively fastidious nature of the cocci and diphtheroids was related to the cutaneous site sampled. Only about 15% of the nasal and toeweb strains grew on medium containing vitamin-free Casitone, whereas essentially all of the groin flora sampled grew on this medium.

Roia and Vanderwyck (16) used skin scruf as a sole nitrogen source in a minimal medium to isolate bacteria from 52 scalp samples. Staphylococci were electively isolated in the medium but the only gram-positive bacilli identified as growing in the medium were sporeforming rods and Brevibacterium species. If one considers the frequent occurrence of corynebacteria on most skin sites (22), it would seem unlikely that this group was not found in any of the 52 samples unless the skin debris was deficient in suitable nutrients to support the growth of corynebacteria from the scalp area.

Urease activity of staphylococci from both oral and cutaneous sites is well known (21), but the results of elective enrichment experiments indicated that urea was not a sole nitrogen source for these cocci. Small numbers of diphtheroids from some skin sites were found that utilized ammonium salts or urea as sole nitrogen sources but were recognized only by elective culture methods. These cutaneous strains, although small in number, are similar to Corynebacterium bovis which also utilizes these compounds for nitrogen (18). Compared with staphylococci or micrococci, cutaneous diphtheroids are notably lacking in urease activity. In this study and those of others, only 10% of a collective total of 670 cutaneous diphtheroids examined for urease activity was positive (6, 12, 19; R. J. Holt, personal communication). The cutaneous anaerobe, C. acnes, is also urease negative (14). The majority of the aerobic or facultative human oral diphtheroids, however, are urease positive (13).

In summary, the gram-positive cocci and diphtheroids of human skin degraded uric acid but did not utilize uric acid as a sole nitrogen source. Ammonium nitrogen did not support growth of cocci, but some diphtheroids grew on this substrate. Cutaneous cocci which are commonly ureolytic did not utilize urea nitrogen. Some diphtheroids utilized urea nitrogen but were generally urease negative. Certain gram-negative bacilli utilized urate or its degradation products, but some of these bacteria also fix nitrogen (5). The majority of the cocci and diphtheroids required amino-nitrogen and vitamins or yeast extract for growth.

Establishment of the exact role of uric acid or any other skin product in regulating the ecological niches of cutaneous bacteria has only begun. Brock (4) pointed out that cultural studies reveal only potential nutrients which isolated strains can utilize but not which substrates the isolates were able to use in their habitat at the time they were isolated. The results of this study, then, must be regarded as only provisional evidence as to the nitrogenous requirements of the major human cutaneous bacteria. Marples (12) criticized current classification schemes of cutaneous diphtheroids because little emphasis has been placed on
habitat-oriented activities of the organisms. Thus, further identification of the bacteria that utilize specific skin products is being made and compared to other existing classification schemes for diphtheroids (6, 12, 19).

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