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ISOLATION AND NUMERICAL TAXONOMIC STUDY OF UREASE-POSITIVE AEROBIC CORYNEBACTERIA FROM LOWER URINARY TRACT OF HEALTHY SWINE

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Urease-positive aerobic corynebacteria were isolated from the prepuce of 50 out of 77 (64.9%) healthy hogs and from the bladder, urethra and vaginal vestibule of 3 out of 10 (30.0%) healthy gilts. A total of 93 isolates from the pigs were divided, based on 41 characteristics, into two groups, A and B. Group A bacteria which were similar each other consisted of 67 isolates from the prepuce of hogs and 3 isolates from the bladder and urethra of gilts. Group B contained the remaining bacteria, which showed great diversity of characters. A numerical taxonomic study of group A strains showed that they were similar, with similarity values of more than 92%, and that they were different from the known species of urease-positive aerobic corynebacteria. It is possible that group A bacteria belong to a new species of genus *Corynebacterium*.

Key words: Corynebacteria, lower urinary tract, healthy swine, numerical taxonomy

INTRODUCTION

There have been several reports of corynebacterial infections in the urinary tract of diseased pigs. In 1954 Weidlich designated his isolates as "*Corynebacterium renale suis*", which were derived from the kidneys of pigs showing embolic purulent nephritis.[12] This species is no longer charcterized and is not included in the Approved List of Bacterial Names.[8] *Corynebacterium suis* was isolated from cases of cystitis and pyelonephritis.[10] Thenceforth cases of cystitis and pyelonephritis in pigs due to *C. suis* have been reported.[5,6,7,9] This bacterium is an anaerobic, catalase-negative short rod, and recent investigation revealed that *C. suis* has the major cell wall component of rhamnose and lysin, and therefore was considered not to belong to genus *Corynebacterium* but to *Eubacterium*.[11]

An attempt was made in the present study to isolate and characterize urease-positive aerobic corynebacteria from the lower urinary tract of healthy hogs and gilts.

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Most of the isolates formed a major group and they were found to be different, by numerical taxonomy, from the known urease-positive aerobic corynebacteria of human and animal origin.

MATERIALS AND METHODS

Pigs examined

Surveys were conducted on 77 and 10 apparently healthy hogs and gilts respectively aged approximately 6 months of Landlace line, slaughtered in the Slaughter House of Ebetsu, Hokkaido, Japan, over a period from September 1983 to March 1984.

Collection of specimens and cultural method

The lower urinary tract of hogs was divided into 3 parts, prepuce, preputial deverticulum and preputial ostium, and the lower urinary tract of gilts was divided into 4 parts, urinary bladder, urethra, vaginal vestibule and vulva. Approximately 1 gram (1×2cm) of mucosal tissue of each part was removed and emulsified in 2ml of nutrient broth. The resulting emulsion was diluted and inoculated on nutrient agar plate. Swabs of each part were also cultured. Incubation was done at 37°C for 2–3 days in aerobic condition.

Selection of corynebacteria

The colonies resembled to those of corynebacteria (0.5–2mm in diameter; round and smooth or rough; yellowish white, white or yellow; opaque or translucent; slightly glistening or dull) were picked up, examined by Gram-stain, and those of Gram-positive straight to slightly curved rods (sometimes short rods or cocobacillary forms) were isolated. When corynebacteria-like colonies grown were apparently similar, one or two representative colonies were randomly selected. When the colonies were not apparently uniform, each representative colony was selected. The number of isolates was, therefore, more than the number of pigs from which urease-positive aerobic corynbabacteria were isolated.

Selection of corynebacteria from the rest of the isolates was done by the following examinations. Production of urease, acid-fast stain, spore formation, metachromatic granules, catalase, oxidase, motility, casein digestion, oxidation or fermentation test, acid from xylose, galactose, trehalose, maltose, lactose, sucrose, mannitol, salicin and inulin, Voges-Proskauer reaction, methyl red reaction, production of indol and hydrogen sulfide, nitrate reduction, nitrite reduction, gelatin liquefaction, hemolytic zones around colonies on blood agar (sheep, guinea pig, horse blood), hydrolysis of Tween 80, starch hydrolysis, esculin hydrolysis, arginine hydrolysis, growth on 8% Nacl added nutrient agar and growth on MacConkey agar. The tests for biochemistry were done as described by Cowan and Steel[2] as described below in "Characters examined

for numerical taxonomy".

Corynebacteria strains examined for grouping and numerical taxonomy

A total of 93 strains isolated from the lower urinary tract of pigs, selected as described above, and thought to be *Corynebacterium* was used for grouping. Of these, 87 were from the prepuce of 50 hogs and 6 were from the urinary bladder, urethra and vaginal vestibule of 3 gilts. Seventy strains which belonged to a major group (group A, as described below) were studied for numerical taxonomy. The known urease-positive aerobic corynebacteria used as reference strains were *C. renale* ATCC 19412, *C. pilosum* ATCC 29592, *C. cystitidis* ATCC 29593, *C. kutscheri* ATCC 15677, *C. pseudotuberculosis* ATCC 19410, *C. pseudodiphtheriticum* ATCC 10700 and *C. hoagii* ATCC 7005. An isolate of unclassified *Corynebacterium*, strain BPF-4, which was isolated from the preputial diverticulum of a healthy hog and sent to the authors' laboratory by J. E. T. Jones, Department of Animal Husbandry and Hygiene, Royal Veterinary College, University of London, Bolton Park, Hertfordshire, England, was also used.

Maintenance of strains

All the isolates were maintained on nutrient agar consisting of beef infusion (400g of beef muscle infused by heat in 1000ml of water), 1% pepton (Polypepton, Daigo Company, Tokyo, Japan), 0.5% Nacl and 1.2% agar (Shoei Company, Tokyo, Japan). They were transfered to the same agar every month.

Characters examined for numerical taxonomy

The following 130 characters, which included those 41 characters used for selection of corynebacteria, were examined for numerical taxonomy.

A. Morphology and cell arrangement

Twenty-four hr cultures on nutrient agar were examined electron microscopically. Length (<0.6, 0.6–0.8, 0.8–1.2, $>1.2\,\mu$m, variable), length / width (1–1.5, 1.5–2.0, 2.0–3.0, >3.0, variable), fimbriae (present, numerous, thick bundles of fimbriae), shape of cell (straight, curved, spiral, coccobacillary, coccal, branching, pleomorphic, square ended, round ended, tapered, fusiform), presence of metachromatic granules and spores, Gram-stain reaction, acid-fast reaction, motility.

B. Colonies

Colonies on the nutrient agar were examined. Growth of colonies visible to the naked eyes (17hr, 36hr). Diameter (<1, 1–2, >2mm), shape of edge (entire, crenated, erose, fimbriate, spreading), elevation (effuse, raised, convex, umbonate), density (transparent, translucent, opaque), pigment formation (yellowish white, white, yellow, pink) examined on 60th hr.

C. Growth in nutrient broth (24 hr incubation)

Surface growth (pellicle or ring formation), even turbidity, flocculent turbidity.

D. Physiology

Growth in nutrient broth at 5°C for 48 days, 42°C for 7 days; growth in the same broth at 37°C within 10 days at initial pH 5.0, 9.0. Autoagglutination at pH3.0, 5.0, 7.0 in citrate buffer.

E. Biochemistry

Tests were done as described by Cowan and Steel[2] (method numbers are given in parentheses): oxidation or fermentation of glucose; acid from 24 sugars (ribose, xylose, arabinose, rhamnose, fructose, galactose, mannose, lactose, maltose, cellobiose, sucrose, trehalose, raffinose, dextrin, starch, inulin, glycogen, mannitol, sorbitol, inositol, erythritol, adonitol, dulcitol, salicin; examined for 14 days) and gas from glucose (examined for 14 days); nitrate reduction (method 1); nitrite reduction; gelatin liquefaction (method 1); esculin hydrolysis (method 1); hippurate hydrolysis (method 1); starch hydrolysis (method 1); arginine hydrolysis (method 1); tyrosine decomposition; hydrolysis of Tween 80, 60, 40, 20 (method 1).

F. Antibiotic and antibacterial sensitivities (concentrations were expressed as those per millilitre of nutrient agar)

Examined for 14 days. Pencillin G (1 unit), streptomycin (2.5 μg), kanamycin (10 μg), gentamycin (0.08 μg), tetracycline 1 μg), neomycin (2 μg), mitomycin C (1 μg), chloramphenycol 10 μg), rifampicin (1 μg), erythromycin (0.02 μg), oleandomycin (0.2 μg), polymyxin B (500 unit), sulfadimetoxine (5mg), methylviolet (0.0005%), sodium oleate (0.1%), sodium azide (0.05%), potassium thiocyanate(4%).

G. Miscellaneous

Tests were done as described by Cowan and Steel[2], unless otherwise stated. Production of indol (method 1) and hydrogen sulfide (SIM agar of Nissui, Tokyo, Japan was used). Production of catalase, oxidase (method 1) and urease (method 1). Voges-Proskauer reaction (method 1), methyl red rection, casein digestion (haloformation) on nutrient agar with 10% of milk added. Growth within 14 days on MacConkey agar, Simmons' citrate agar and 8% NaCl added nutrient agar. Growth in malonate medium. Hemolytic zones around colonies on blood agar (sheep, guinea pig, horse blood).

Numerical taxonomic analysis

Analysis of the results obtained with total 70 strains of group A was performed by the method of Lessel and Holt.[4] Thirty-four tests where all strains gave negative or positive results were not incorporated in the data table. After calculation of similarity (S) values, the strains were clustered by single linkage in which each strain was admitted to a group at the highest similarity level it had with any member of that group.

Diagrammatic representation of the grouping of the strains was done by means of

a dendrogram which made apparent the affinities of the strains and clusters of strains.

Results

Isolation of urease-positive aerobic corynebacteria from the lower urinary tract of pigs

Seven hogs preliminarily examined showed that urease-positive aerobic corynebacteria from the lower urinary tract were isolated most frequently from the prepuce (4 out of 7 hogs), but not so from neither the preputial diverticulum (1 out of 7 hogs) nor the preputial ostium (none of 6 hogs; a specimen from the remaining hog was contaminated and unable to examine). Therefore, successive isolation of the bacteria from hogs was made only from the prepuce.

The rate of isolation of urease-positive aerobic corynebacteria from the prepuce of randomly selected 20 hogs was compared between the tissue emulsion and swab. The results showed that the rate of isolation was higher from the tissue emulsion (16 out of 20 hogs, 80%) than from the swab (8 out of 18 hogs, 44%; 2 swab were contaminated with swarming bacteria and unable to examine).

Fifty (64.9%) out of 77 hogs examined were found to have urease-positive aerobic corynebacteria in the prepuce (Table 1). Urease-positive corynebacteria were also isolated from the lower urinary tract of gilts, but less frequently (3/10, 30%).

The number of bacteria isolated from the prepuce were less than 10 bacteria per 0.01 gram tissue in 28 hogs (56.0%), 10 to 100 bacteria in 13 hogs (26.0%) and 100 to 1000 bacteria in 9 hogs (18.0%).

A total of 93 strains were selected as described in "Selection of corynebacteria" in Materials and Methods. The number of strains which were positive for each

Table 1 Isolation of uresae-positive aerobic corynebacteria from lower urinary tract of pigs

Pigs	Isolation of urease-positive aerobic corynebacteria[a]	Per cent
Hogs[b]	50/77	64.9
Gilts[c]	3/10	30.0

[a] The denominator shows the number of pigs examined. The numerator denotes the number of pigs from which urease-positve aerobic corynebacteria were isolated.

[b] Urease-positive aerobic corynebacteria were isolated from the prepuce.

[c] Urease-positive aerobic bacteria were isolated from the urinary bladder (2 gilts), urethra (1 gilt) and vaginal vestibule (2 gilts).

character was as follows (in parenthesis, the numerator shows the number of positive strains while the denominator denotes the number of strains examined). Colonies (60th hr) : 1–2mm in diameter (73/93) ; round and smooth (86/93) ; slightly glistening (most strains) ; yellowish white (77/93). Rods with club-shaped forms (70/93). Gram-stain (93/93). Acid-fast stain (2/93). Spore formation (0/93). Metachromatic granules (72/93). Catalase (93/93). Urease (93/93). Oxidase (0/93). Motility (0/93). Oxidation or fermentation test (93/93 fermentive). Acid from xylose (60/93), galactose (72/93), trehalose (69/93), maltose (74/93), sucrose (70/93), lactose (only a few positive), salicin (only a few positive), mannitol (0/93). Voges-Proskauer reaction (4/93). Methyl red reaction (3/93). Indol production (0/93). Hydrogen sulfide production (0/93). Nitrate reduction (89/93). Nitrite reduction (1/93). Gelatin liquefaction (0/93). Hemolysis of red cell of sheep (0/93), guinea pig (0/93), horse (1/93). Hydrolysis of Tween 80 (69/93). Hydrolysis of starch (74/93). Esculin hydrolysis (1/93). Arginine hydrolysis (0/93). Growth on 8% Nacl added nutrient agar (5/93). Growth on MacConkey agar(0/93).

The above characters indicated that the 93 strains tested met the difinition of *Corynebacterium*[1] except Mol% G+C and cell wall composition.

Grouping of isolates

Eighty-seven isolates from 50 hogs, together with 6 isolates from 3 gilts, were examined for characteristics listed above in "Selection of corynebacteria" in Materials and Methods. It was shown that 70 of the 93 isolates (group A) were similar in a number of characters (Table 2). The remaining 23 strains showed characters different from those of group A of which many characters were diverse among strains. These bacteria were tentatively grouped as B. The bacteria of group A were straight to slightly curved rods with irregularly stained segments, and often contained metachromatic granules, produced acid from 5 sugars, xylose, galactose, trehalose, maltose and sucrose, and hydrolyzed Tween 80 and starch. Of the 70 strains of group A, 67 were isolated from 47 hogs and 3 were from 1 gilt. Group B bacteria were short in length, did not produce acid from these sugars nor hydrolyze Tween 80.

Numerical taxonomic analysis of the group A bacteria

Group A bacteria, together with the known urease-positive corynebacteria, examined for 130 features, were subjected to numerical taxonomic analysis. Twenty-nine characters (length variable, length / width variable, curved cell, spiral cell, coccal cell, branching cell, pleomorphic cell, square ended cell, fusiform cell, spore formation, motility, convex colony, erose colony, fimbriate colony, spreading colony, transparent colony, gas from glucose, acid from lactose, sorbitol, inositol and erythritol, arginine hydrolase, decomposition of tyrosine, oxidase test, production of hydrogen sulfide and indol, hemolytic zones around colonies on sheep and guinea pig blood agar, and growth

TABLE 2 Grouping of urease-positive aerbic corynebacteria isolated from lower urinary tract of pigs

Characteristics	Group A	Group B
Shape of cell (μm)	0.18–1.4 x 0.4–0.6	0.6–0.9 x 0.5–0.8
Pigment (nondiffusible)	Yellowish white	V[a]
Acid from xylose	+[b]	−
galactose	+	−
trehalose	+	(−)[c]
maltose	+	(−)
sucrose	+	−
Caseinase	−	(−)
Nitrate reduction	+	(+)
Hydrolysis of Tween 80	+	−
Hydrolysis of starch	+	V
Number of isolates from		
hogs	67	20
gilts	3[d]	3

[a] V means that the characteristic for each strain was various.
[b] + or − without parenthesis means that more than 80% of strains of the group showed the same characteristics.
[c] ( ) means that more than 60% of strains of the group showed the same characteristics.
[d] Isolated from the bladder and urethra of gilts.

on MacConkey agar) which were negative for every strain and 5 characters (Gram-stain reaction, colony visible within 36 hr, catalase test, urease test, and growth in the presence of erythromycin 0.02 μg) which were positive for every strain were not used in the numerical analysis. The remaining 96 characters in which there were strain differences were used in the numerical analysis. The group A strains which were identical or very similar to each other were represented by a strain arbitarily selected, thus 30 representative strains are on the dendrogram (Fig. 1).

These strains were closely similar to each other, with a similarity value of more than 92%, except one strain (31–23), and were different from the known species of urease-positive aerobic corynebacteria, *C. renale*, *C. pilosum*, *C. cystitidis*, *C. kuts-*

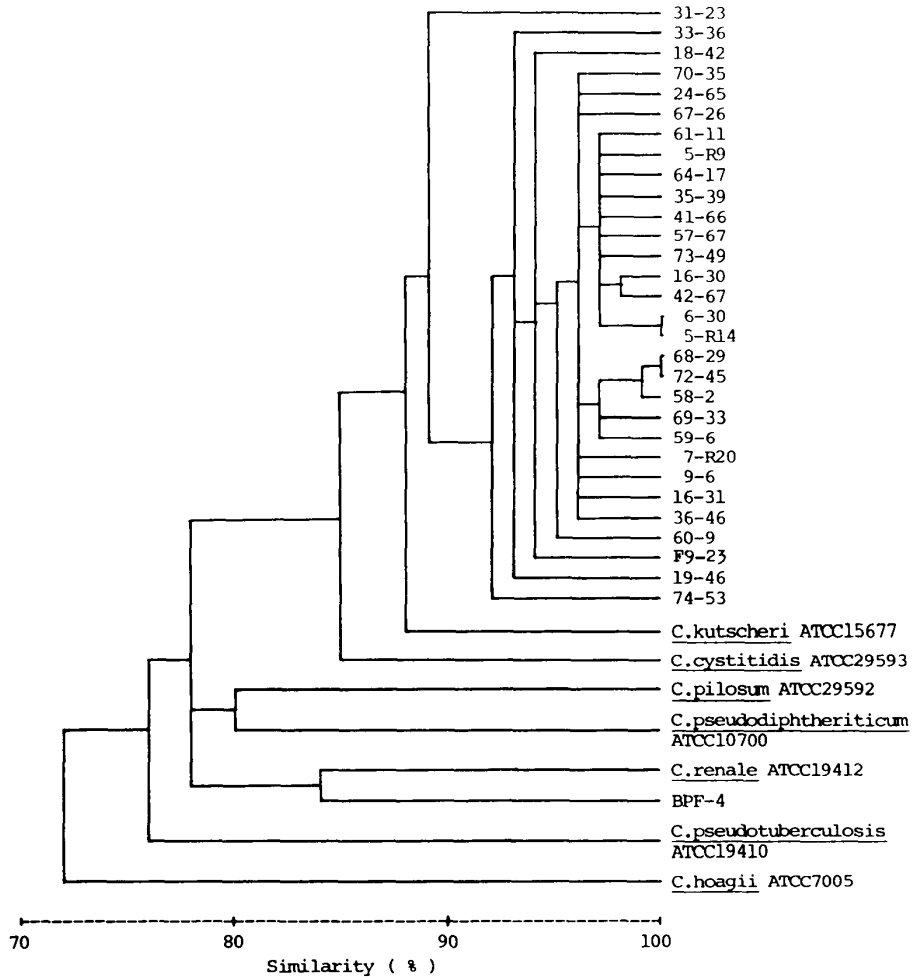

Fig. 1 Dendrogram obtained from similarity values for the strains of group A

cheri, *C. pseudotuberculosis*, *C. pseudodiphtheriticum*, *C. hoagii* and an unclassified corynebacterial strain BPF-4. The bacteria of group A were, therefore, supposed to be a new species of genus *Corynebacterium*.

Strain 5-R14 was considered to be a representative of the group A bacteria. Table 3 shows characteristics in which there are differences among strain 5-R14 and 7 species of the known urease-positive aerobic *Corynebacterium*.

TABLE 3 Characteristics in which there are strain differences

Characteristics	Strains[a] with positive reaction
Cell morphology	
Length (0.8–1.2 μm)	A, h, k, pd, pt, r
Length (>1.2 μm)	c, p
Length/width (1.5–2.0)	p, pd, pt
Length/width (2.0–3.0)	A, h, k, r
Length/width (>3.0)	c
Straight cell	A, c, k, pd, r
Cocco-bacillary cell	h, p, pt
Metachromatic granules	A, c, k, r
Fimbriae (present)	A, c, h, k, p, pd
Fimbriae (numerous)	A, c, h, k, p
Fimbriae (thick bundle formation)	A, c, k, p
Colony	
Size (<1 mm)[b]	c
Size (1–2 mm)[b]	A, k, p, pd, pt, r
Size (>2 mm)[b]	h
Nondiffusible pigment (white)	c, k, pd, pt
Nondiffusible pigment (yellowish white)	A
Nondiffusible pigment (yellow)	p, r
Nondiffusible pigment (pink)	h
Growth in nutrient broth	
Surface growth	h, pd, pt
Temperture of growth in nutrient broth	
Growth at 42° C for 7 days	r
pH growth range in nutrient broth	
Growth at initial pH 5.0	h
Growth at initial pH 9.0	c, h, p, pd, r
Spontaneous agglutination in	
Citrate buffer (pH 3.0)	A, c, k, p, pd, pt, r
Citrate buffer (pH 5.0)	k
Biochemistry	
Glucose, aerobic tube acid	A, c, k, p, pt, r
Glucose, anaerobic tube acid	A, c, k, p, pt, r
Acid from :	
Ribose	A, k, r
Xylose	A, c
Rhamnose	A
Fructose	A, k, r
Galactose	A, k, pt
Mannose	A, k, pt, r
Maltose	A, k, pt
Sucrose	A, k
Trehalose	A, r
Dextrin	A, k

Characteristics	Strains[a] with positive reaction
Starch	A
Inulin	k, pt
Mannitol	k, pt
Salicin	k, pt
Starch hydrolysis	A, c, p, pt
Esculin hydrolysis	k
Hippurate hydrolysis	A, c, k, p, r
Nitrate reduction	A, h, k, p, pd
Nitrite reduction	p, pd, pt
Gelatin liguefaction	p
Hydrolysis of Tween 80	A, c, h, pd
Hydrolysis of Twenn 60	A, c, h, pt
Hydrolysis of Tween 40	A, c, h, k, p, pt, r
Hydrolysis of Tween 20	A, c, h, k, p, pd, r
Casein digestion	r
Methyl red reaction	pt, r
Growth in malonate medium	h
Antibiotic and antibacterial sensitivities	
Penicillin (1 unit)	c, h, k
Streptomycin (2.5 μg)	A, p, r
Kanamycin (10 μg)	h
Gentamycin (0.08 μg)	h, p, pd, pt
Tetracycline (1 μg)	A, h, p, r
Oleandomycin (0.2 μg)	A, c, h, k, pd, pt, r
Mitomycin (1 μg)	h
Rifampicin (1 μg)	h
Polymyxin B (500 unit)	h, pt, r
Nacl (8 %)	p, pd, r
Sodium oleate (0.1 %)	h, p, r
Potassium thiocyanate (4 %)	r
Sodium azide (0.05 %)	h

[a] Symbols : A, strain 5–R 14 (gropup A); c, *C. cystitidis* ATCC 29593 ; h, *C. hoagii* ATCC 7005 ; k, *C. kutscheri* ATCC 15677 ; p, *C. pilosum* ATCC 29592 ; pd, *C.pseudodiphtheriticum* ATCC 10700 ; pt, *C. pseudtuberculosis* ATCC 19410 ; r, *C.renale* ATCC 19412.

[b] Diameter of colonies measured at 60 th hr.

Discussion

Urease-positive aerobic corynebacteria were frequently isolated from the prepuce of nearly 65% of 77 healthy hogs examined. A large number of the isolates from hogs and 3 isolates from 1 gilt were similar to each other in many features and grouped together (group A). A numerical taxonomic study of group A bacteria, which examined with 130 characters, showed that these strains formed a cluster of which the similarity value was different from the known species of urease-positive aerobic *Corynebacterium*, and thus were thought to be a new species of genus *Corynebacterium*. A representative strain (5–R14) is being deposited in the American Type Culture Collection under the tentative designation of *Corynebacterium hyourinaetractum*. However, further studies on DNA homology, Mol% G+C and cell wall composition are necessary to define its taxonomic position.

The bacteria are considered to be normal flora of the prepuce of hogs, as no macroscopic lesions were found in the prepuce. They seem to be normal flora of the prepuce of hogs like *C. cystitids*, which is a member of the normal flora of the prepuce of bulls.[3)]

The group of bacteria were also isolated from the lower urinary tract of gilts. It is unknown whether these bacteria are pathogenic in gilts or sows, which will be a subject of a future study.

Differential characteristics: Table 4 contains a list of properties that are useful in differentiating strain 5–R14, a representative of group A bacteria, from the other species of urease-positive aerobic *Corynebacterium* that are parasitic on or pathogenic to humans and / or other animals.

TABLE 4 Characteristics useful in differentiating strain 5–R14 (group A) from other species of urease-positive aerobic corynebacteria that are parasitic on or pathogenic to human and other animnals

Characteristics	Results obtained with							
	5–R14 (group A)	*C. kutscheri* ATCC 15677	*C. cystitidis* ATCC 29593	*C. pilosum* ATCC 29592	*C. pseudodiphtheriticum* ATCC 10700	*C. renale* ATCC 19412	*C. pseudotuberculosis* ATCC 19410	*C. hoagii* ATCC 7005
Acid from xylose	+	−	+	−	−	−	−	−
arabinose	−	−	−	−	−	−	−	−
rhamnose	+	−	−	−	−	−	−	−
fructose	+	+	−	−	−	+	−	−
galactose	+	+	−	−	−	−	+	−
mannose	+	+	−	−	−	+	+	−
maltose	+	+	−	−	−	−	+	−
sucrose	+	+	−	−	−	−	−	−
trehalose	+	−	−	−	−	+	−	−
dextrin	+	+	−	−	−	−	−	−
starch	+	−	−	−	−	−	−	−
Starch hydrolysis	+	−	+	+	−	−	+	−
Esculin hydrolysis	−	+	−	−	−	−	−	−
Hippurate hydrolysis	+	+	+	+	−	+	−	−
Nitrate reduction	+	+	−	+	+	−	−	+
Nitrite reduction	−	−	−	+	+	−	+	−
Hydrolysis of Tween 80	+	−	+	−	+	−	−	+
Caseinase	−	−	−	−	−	+	−	−
Sodium oleate	−	−	−	+	−	+	−	+

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