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YOKENELLA REGENSBURGEI GEN. NOV., SP. NOV.:

A NEW GENUS AND SPECIES IN THE FAMILY ENTEROBACTERIACEAE

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SUMMARY: The name Yokenella gen. nov. is proposed for a group of organisms in the family Enterobacteriaceae isolated from clinical sources and insects. Yokenella is a gram-negative, oxidase-negative, fermentative, motile rod possessing the characteristics of the family Enterobacteriaceae and the guanine plus cytosine contents of the DNA range from 58.0 to 59.3 mol%. Biochemical characteristics of this group and DNA hybridization studies indicate that the 11 strains studied here comprise a separate species which should be best placed in a new genus. This single DNA hybridization group is named Yokenella regensburgei sp. nov. The type strain of Y. regensburgei is NIH 725-83 (JCM 2403).

INTRODUCTION

Since 1965, the Enterobacteriology Laboratories in the National Institute of Health (NIH), Tokyo, received many strains that did not belong to any recognized species of Enterobacteriaceae. These strains are divided into several biogroups with vernacular names, one of them is called NIH biogroup 9, and kept in the collection of the laboratories for the purpose of detecting further strains of similar unspecified bacteria. In 1982 and 1983, one of the present authors (R. S.) received 30 strains resembling Hafnia alvei from Dr. Friedrich Haas, Regensburg, the Federal Republic of Germany, who is studying the intestinal flora of insects, Heteroptera and Coleoptera, with his request for confirming his identification. After extensive examinations at NIH, 24 of the 30 strains were identified as Hafnia alvei on the basis of overall biochemical reactions and the susceptibility to Hafnia phage 1672 of Guinea and Valkenburg (1). The remaining six were found to be different from H. alvei in their negative reactions for Voges-Proskauer and the resistance to Hafnia phage. Computer identification, described by Lapage et al. (2), for the six strains based on 24 biochemical test results has revealed that these strains possess a similarity of 0.99 to NIH biogroup 9 comprising five clinical isolates but less

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than 10^{-5} to Hafnia alvei, suggesting that the six strains are included in the same taxon of NIH biogroup 9. In addition, further biochemical characterization and DNA studies on the 11 strains of NIH biogroup 9 let the present authors place them in a new species belonging to a new genus. In this paper, the names Yokenella gen. nov. and Yokenella regensburgei sp. nov. are proposed for this group of organisms.

MATERIALS AND METHODS

Strains studied: the Yokenella regensburgei strains used in this study are listed in Table I. Of the 11 strains in the table, the five originating from clinical specimens were sent to the Enterobacteriology Laboratories, NIH, from clinical microbiology laboratories in several regions in Japan. The other six strains were isolated from the middle gut of Heteroptera (Pyrrhcoris apterus) by Dr. F. Haas. The seven reference strains other than Yokenella used in the DNA relatedness study are listed in Table III.

Biochemical tests: The following tests were performed as described by Edwards and Ewing (3) and Cowan (4). The tests were: oxidase by Kovacs method; reduction of nitrate to nitrite; H_2S production in the butt of Kligler iron agar (BBL) and peptone iron agar (Difco); urea decomposition on Christensen agar; citrate utilization on Simmons agar; acetate utilization; phenylalanine deaminase; malonate utilization; motility in motility test medium at 37 C; gelatine liquefaction by Kohn method; decarboxylation of lysine and ornithine and dehydrolation of arginine in Møller broth (Difco); deoxyribonuclease on DNase test agar (BBL) with 0.01% toluidine blue at 25 C for 6 days; hydrolysis of tween 80 and corn oil within 7 days; D-tartrate and mucate decomposition in Kauffmann-Peterson medium (Difco) within 4 days; pectinase on polypectate agar plate; gas production from glucose in phenol red broth (Difco) with a Durham tube. Voges-Proskauer test was performed by the method modified by Richard (5).

Table I. *Sources of the strains studied*

Strains ^a	Isolated from;
1251-72	Human, abscess
361-78	Human, urine
111-79	Human, urine
9-72	Human, urine
963-80	Human, wound
725-83	Insect, intestine
726-83	Insect, intestine
727-83	Insect, intestine
728-83	Insect, intestine
729-83	Insect, intestine
730-83	Insect, intestine

a: Strains isolated from human specimens were obtained from clinical microbiology laboratories in several locations in Japan and from insect (Pyrrhcoris apterus) were provided by Dr. F. Haas, Federal Republic of Germany.

For carbohydrate fermentation, API 50CH was used. Pigmentation was examined on heart infusion agar at 25 C for 15 days. β -Xylosidase (ONPX) was tested by the method of Brisou et al(6). Tetrathionate reduction test was performed as described by Le Minor et al(7). For the test of β -galactosidase, ONPG disc (Nissui) was used.

Preparation of unlabeled DNA: The bacteria were grown to mid-log phase in 300 ml of brain heart infusion broth in an orbital incubator (New Brunswick) at 30 C. The cells were sedimented in a refrigerated centrifuge, washed with saline-EDTA (0.15 M NaCl and 0.1 M ethylenediaminetetraacetic acid disodium salt) and resuspended in the same solution. Lysis of the cells was accomplished by adding sodium lauryl sulfate (SLS) to 1% (v/v) at 60 C. An equal volume of phenol was added to the cell lysate and the resulting aqueous phase separated by centrifugation at 12,000 g for 10 min at 5 C, was carefully collected. Two volumes of cold 95% ethanol were added to the aqueous phase. The DNA precipitate was then spooled on a glass rod and dissolved in 10 ml of SSC (0.15 M NaCl and 0.015 M trisodium citrate). RNase was added to the DNA solution, which was incubated at 37 C for 30 min followed by addition of an equal volume of phenol to extract again. After repeated precipitation and spool on a glass rod, the DNA was finally dissolved in SSC. Solution was assayed spectrophotometrically for purity and concentration as described by Marmur(8) and Marmur and Doty(9) and stored at 4 C with chloroform.

Preparation of radiolabeled DNA: Radiolabeling of DNA was carried out by the in vitro nick translation method (10) with a commercial reagent kit (catalog No. NEN-005, New England Nuclear).

Determination of base composition of DNA: The guanine plus cytosine contents of DNA were determined from the thermal denaturation temperature by the method of Owen et al. (11).

DNA hybridization: The relatedness of labeled DNA to unlabeled DNA was determined by hybridization on nitrocellulose filter according to Johnson (10), Denhardt (12) and Suzuki et al (13).

RESULTS

Phenotypic characterization: The 11 strains of NIH biogroup 9 are gram-negative, oxidase-negative, catalase-positive, non-sporeforming, fermentative

Fig. 1. Electron micrograph (x17,000) of a cell and flagella of *Y. regensburgeri* type strain NIH 725-83 (JCM 2403).

rods. Of the 11 strains, 10 are motile with peritrichous flagella (Fig. I).

The reactions of the 11 strains in physiological and biochemical tests are shown in Table II. As shown in the table, they gave positive reaction in the tests for lysine and ornithine decarboxylases, utilization of citrate as a sole carbon source, KCN and ONPG. They gave negative Voges-Proskauer reaction and did not produce indole, H₂S, urease, phenylalanine deaminase, gelatinase, DNase, lipase, or β -xylosidase. They fermented glucose, L-arabinose, cellobiose, melibiose, rhamnose and mannitol with production of acid and gas. Lactose,

Table II. *Biochemical characteristics of NIH biogroup 9 (Yokenella regensburgeri)*

Test (Substrate)	Sign %+	Test (Substrate)	Sign %+
Oxidase	- 0	Acid from: (Continued)	
Hydrogen sulfide (Kligler)	- 0	L-Fucose	+ 100
(PIA)	- 0	D-Fucose	- 0
Indole	- 0	β -Gentibiose	+ 100
Voges-Proskauer (37 C)	- 0	Lactose	- 0
(25 C)	- 0	D-Levulose	+ 100
Citrate (Simmons)	+ 100	Lyxose	- 0
(Christensen)	+ 100	Maltose	+ 100
Motility	+ 91	D-Mannose	+ 100
KCN growth	+ 100	Melibiose	+ 100
Growth at 4 C	- 0	Melezitose	- 0
Pigmentation (yellow)	- 0	Raffinose	- 0
Lysine decarboxylase	+ 100	L-Rhamnose	+ 100
Arginine dihydrolase	- 0	L-Sorbose	- 0
Ornithine decarboxylase	+ 100	Sucrose	- 0
Phenylalanine deaminase	- 0	D-Tagatose	- 0
Gelatin	- 0	Trehalose	+ 100
DNase	- 0	D-Turanose	- 0
Lipase (Corn oil)	- 0	D-Xylose	+ 100
(Tween 80)	- 0	Adonitol	- 0
Malonate (Ewing)	- 0	D-Arabitol	- 0
Urease (Christensen)	- 0	Erythritol	- 0
D-Tartrate (K-P)	- 0	Dulcitol	- 0
Acetate	- 0	myo-Inositol	- 0
Tetrathionate reductase	+ 100	Mannitol	+ 100
Pectate	- 0	Sorbitol	- 0
Chitinase	- 0	Amygdaline	- 0
Growth on		arbutine	- 0
Caprylate-thallos agar	- 0	α -Methyl-D-glucoside	- 0
β -Galactosidase	+ 100	Salicin	- 0
β -Xylosidase	- 0	Xylitol	- 0
D-Glucose, acid	+ 100	Gluconate	+ 100
gas	+ 100	2-Keto-gluconate	- 0
Acid from:		5-Keto-gluconate	- 0
L-Arabinose	+ 100	Esculin hydrolysis	- 0
Cellobiose	+ 100		

Reaction obtained within 48 h at 36 $\pm$ 1 C unless specified; +, 90% or more positive; -, 0 to 9.9% positive.

raffinose, sucrose, adonitol, myo-inositol, sorbitol, α -methyl-D-glucoside or salicin was not attacked. They failed to grow at 4 C and on the caprylate-thallos agar. No strain produced pigment. Further characteristics of the 11 strains are given in the table.

G+C contents of DNA: The G+C contents of DNA from six strains ranged from 58.0 to 59.3 mole%, well within the range for Enterobacteriaceae.

DNA relatedness: DNA from a representative strain of NIH biogroup 9 was radiolabeled and tested for relatedness with the other strains of the same biogroup and with some species of genera Escherichia, Citrobacter, Enterobacter and Hafnia. The results are shown in Table III. Table III includes also the results obtained with labeled DNA from Hafnia alvei ATCC 13337. The degree of reassociation between labeled DNA from strain 728-83 and unlabeled DNA from other strains of the same biogroup ranged from 89 to 100% at 65 C and from 87 to 100% at 75 C. Strain 728-83 exhibited 35 to 42% relative binding with Enterobacter cloacae, Escherichia coli, and Citrobacter freundii, 24 and 27% with Hafnia alvei and Escherichia adecarboxylata, respectively and 9 and 12% with Enterobacter agglomerans and Escherichia hermannii, respectively at 65 C. In the more stringent incubation at 75 C, less than 25% reassociation occurred between DNA from strain 728-83 and that from other species tested. On the other hand, relatedness between labeled DNA from H. alvei and unlabeled ones from eight strains of NIH biogroup 9 ranged from 19% to 25% at 65 C.

Table III. *DNA relatedness of 725-83 to other strains of NIH biogroup 9 and to other species of Enterobacteriaceae*

Strains	%Homology			
	G+C	<u>H. alvei</u>	725-83	
	(%)	65 C	65 C	75 C
725-83	59.0	19	100	100
726-83	58.0	22	89	88
727-83	58.8	23	97	96
728-83	59.3	23	100	100
729-83	59.0	25	99	98
730-83	58.9	24	100	100
1251-72	58.5	25	89	87
111-79	59.0	24	95	92
<u>Escherichia coli</u> , ATCC 11775	ND	ND	42	23
<u>Escherichia adecarboxylata</u> , ATCC 23216	ND	ND	27	15
<u>Escherichia hermannii</u> , ATCC 33650	ND	ND	12	4
<u>Citrobacter freundii</u> , ATCC 8090	ND	ND	42	24
<u>Enterobacter cloacae</u> , ATCC 13047	ND	ND	35	19
<u>Enterobacter agglomerans</u> , ATCC 27155	ND	ND	9	2
<u>Hafnia alvei</u> , ATCC 13337	52.0	100	24	7

ATCC: American Type Culture Collection, Rockville, Md.; ND : Not determined.

DISCUSSION

The DNA relatedness study showed that eight strains of NIH biogroup 9 consist a single DNA hybridization group distinct from genera Escherichia, Citrobacter, Enterobacter and Hafnia in the family Enterobacteriaceae.

When the 11 strains of NIH biogroup 9 were tested with four commercial identification systems, two (Micro-ID, General Diagnostic and Enterotube II, Roche) of the systems identified them as H. alvei and one (Minitek, BBL) identified them as Salmonella arizonae, whereas the numerical profile of them given by the remaining system (API 20E, API System) was not found in its own profile index. Strains of this biogroup are different from H. alvei in Voges-Proskauer test, citrate utilization, acid production from melibiose and 2-keto-gluconate and the ability to grow at 4 C. In addition to these, the G+C contents of DNA of NIH biogroup 9 is approximately 59 mol%, whereas that of H. alvei is 52 mol%. NIH biogroup 9 is distinguished from Salmonella arizonae in the positive growth in the presence of KCN and fermentation of cellobiose and the negative tests for H₂S and malonate and fermentation of sorbitol and 5-keto-gluconate. Tests useful in differentiating NIH biogroup 9 from other species of Enterobacteriaceae resembling to this group are shown in Table IV. NIH biogroup 9 is distinguished from members of Cedecea, Kluyvera, Serratia, Citrobacter amalonaticus, Citrobacter diversus, Ewingella americana, Buttiauxella agrestis and some species of Enterobacter in its positive reaction in lysine and ornithine decarboxylases and negative reactions in Voges-Proskauer, DNase, lipase, gelatinase, ONPX, pigmentation, fermentation of lactose, sucrose, raffinose, adonitol and sorbitol.

Brenner(14) presented three hybridization groups of H. alvei. He found several differential characters as follows; they give negative reactions in Voges-Proskauer, D-tartrate, urease and D-levulose, and positive reactions in acetate utilization and fermentation of salicin, arbutine and esculin; and the third DNA relatedness group is resistant to Hafnia-specific phage. On the other hand, NIH biogroup 9 shows positive reactions for levulose and negative reaction for salicin, arbutin esculin and acetate utilization. Thus NIH biogroup 9 could well be separable from the third group of H. alvei.

From these results, the present authors concluded that NIH biogroup 9 is a new species belonging to a new genus and propose the name Yokenella regensburgei for this group of organisms.

Description of the genus Yokenella:

Yokenella gen. nov. is an arbitrarily constructed name derived from the Japanese abbreviation "Yoken" for the National Institute of Health, Tokyo, and a modern Latin feminine noun formed by adding the diminutive ending "-ella" to the

Table IV. *Differentiation between Yokenella regensburgei and other species of Enterobacteriaceae*

Test	Yoken- ella regens- burgei	Esche- richia coli	Esche- richia vulne- ris	Salmon- ella arizonae	Citro- bacter freundii	Citro- bacter amalo- naticus	Citro- bacter diver- sus	Entero- bacter aero- genes	Entero- bacter sakaza- kii	Entero- bacter gergovi- ae	Hafnia alvei	Cedecea spp.	Kluyvera spp.
Indole	-	+	-	-	-	+	+	-	-	-	-	-	d
Voges-Proskauer (37 C)	-	-	-	-	-	-	-	+	+	+	d	d	-
Lysine decarboxylase	+	+	d	+	-	-	-	+	-	+	+	-	-
Ornithine decarboxylase	+	-	-	+	d	+	+	+	+	+	+	d	+
Citrate (Simmons)	+	d	-	+	+	d	+	+	+	+	-	+	+
Lipase (Tween 80)	-	-	-	-	-	-	-	-	-	-	-	+	-
KCN	+	-	d	-	+	+	-	+	+	-	+	+	+
Acid from;													
L-Arabinose	+	+	+	+	+	+	+	+	+	+	+	-	+
Cellobiose	+	-	+	+	d	+	+	+	+	+	-	+	+
Melibiose	+	d	+	+	d	d	-	+	+	+	-	+	+
Adonitol	-	-	-	-	-	-	+	+	-	-	-	-	-
D-Arabitol	-	+	-	-	+	+	d	-	-	+	d	-	d
Sorbitol	-	+	-	+	+	+	+	+	-	-	+	d	d
α-Methyl-D-glucoside	-	-	d	-	-	d	d	+	+	-	-	d	d
Esculin	-	d	d	-	-	-	-	+	+	+	-	+	+
2-Keto-gluconate	-	-	+	-	+	+	+	+	+	+	+	+	+
5-Keto-gluconate	-	d	-	+	+	+	+	-	+	+	-	-	+
Yellow pigment	-	-	d	-	-	-	-	-	+	-	-	-	-

Reactions were tested at 36±1 C; +, 90% or more positive within 48 hr; -, 0 to 9.9% positive within 48 hr; d, 10.0 to 89.9% positive within 48 hr.

noun "Yoken". The specific epithet is a modern Latin genitive, regensburgei, pertaining to Regensburg (the Federal Republic of Germany) where the type strain of this species was isolated from insects.

The proposed genus Yokenella is monotypic and a description of the genus is also given as that of species, Yokenella regensburgei sp. nov.

Gram-negative, oxidase-negative, non-sporeforming, motile rods with peritrichous flagella. Not pigmented. It reduces nitrate to nitrite and ferments glucose and other carbohydrates with the production of acid and gas. Grows at 37 C, but not at 4 C. As a sole carbon source, citrate is utilized but not malonate. Gives a negative Voges-Proskauer reaction. Grows in KCN broth. Lysine and ornithine decarboxylases and β -galactosidase are produced. L-arabinose, cellobiose, levulose, melibiose and L-rhamnose are fermented. Acid is not produced from lactose, raffinose, adonitol, D-arabitol, dulcitol, D-sorbitol, myo-inositol, arbutin, salicin, α -methyl-D-glucoside, 2-keto-gluconate or 5-keto-gluconate. Esculin is not hydrolyzed.

G+C contents of DNA is 59 mol%.

The type strain is NIH 725-83 (JCM 2403) isolated from the intestine of insects.

Found in man and insects. The clinical significance of Y. regensburgei in man is unknown.

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