Identification of the Psychrophilic Histamine-Producing Marine Bacteria Previously Referred to as the N-group Bacteria

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The taxonomic position of the psychrophilic histamine-producing marine bacteria previously referred to as the N-group bacteria, were studied phenotypically and genotypically. These strains were facultatively anaerobic Gram-negative asporogenous coccobacilli, which were motile by means of un-sheathed polar flagella. They were also positive for D-glucose fermentation and poly- β -hydroxybutyrate accumulation, and had guanine plus cytosine contents of genomic DNA of 40 to 43 mol%. The N-group bacteria exhibited high levels of genomic DNA hybridization homology to the type strain of *Photobacterium phosphoreum*. The phenotypic and genotypic data suggested that the N-group bacteria are members of *P. phosphoreum*.

Key words: histamine, marine bacteria, *Photobacterium phosphoreum*

Histamine is generally found in spoiled scombroid fish and other marine fish that contain high levels of histidine in their muscle tissues,¹⁾ and is associated with the chemical intoxication called scombroid fish poisoning. Many workers isolated various histamine-producing bacteria from the fish which had been involved in food poisoning incidents.²⁾

Most of the histamine-producing bacteria isolated so far have been identified as being the members of the family Enterobacteriaceae.²⁾ On the other hand, Okuzumi *et al.*³⁻¹⁰⁾ isolated two groups of histamine-producing marine bacteria with respect to growth temperatures. One of these groups included mesophiles for which we recently proposed to create a new species with the name *Photobacterium histaminum* sp. nov.³⁾ The other group included psychrophiles which grew well at 4°C but not at 40°C and were tentatively named the N-group bacteria.⁴⁾ While the ecological studies of the N-group bacteria in marine fish^{5,7,8)} and sea environment⁶⁾ have been made, the taxonomic position of these bacteria have not been studied in detail.

In the present report, we described the characterization and identification of the N-group bacteria phenotypically and genotypically.

Materials and Methods

Bacterial Strains

Six histamine-producing strains which were isolated from marine fish and the sea water of Sagami Bay in Japan and have been previously referred to as the N-group bacteria were used. Among them, strains N-XBO (from horse

mackerel), N-KGF (from common mackerel) and N-VEF (from sea water) were luminous, and strains N-YAG (from sardine), N-MHL (from sea water) and N-611A (from saury pike) were nonluminous. *Photobacterium phosphoreum* ATCC 11040^T, *P. leiognathi* ATCC 25521^T, *P. angustum* ATCC 25915^T and *P. histaminum* JCM 8968^T were used as the reference strains for DNA-DNA homology.

Phenotypic Characterization

Morphological, physiological and biochemical characteristics were studied as described previously.³⁾ Except where stated otherwise, cultivation temperature for the strains was 20°C, and the media were prepared with 75 percent artificial sea water.¹¹⁾

Genetic Properties

Genomic DNA was isolated and purified in accordance with the method of Marmur,¹²⁾ and moles percent guanine plus cytosine (G+C) of DNA was determined by the HPLC method.¹³⁾ Experiments for DNA-DNA relatedness were carried out by the dot blot hybridization method with photobiotin labeling and colorimetric detection as described previously.¹⁴⁾

Results

The morphological, physiological and biochemical characteristics of the tested strains are shown in Table 1. They were Gram-negative asporogenous coccobacilli, 1.0~1.2 μ m in diameter and 2.0~2.2 μ m in length. These strains were motile by means of 1 to 3 polar flagella. Electron

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Table 1. Characteristics of the histamine-producing bacteria

Characteristics	Luminous 3 strains ^a	Nonluminous 3 strains ^b
Gram stain	—	—
Coccoid rod	+	+
Spore	—	—
Motility	+	+
Sheathed polar flagella	—	—
Acid from D-glucose	+	+
Gas from D-glucose	+	+
Catalase	+	+
Oxidase	(1)	(2)
Reduction of NO ₃ ⁻ to NO ₂ ⁻	+	+
Accumulation of PHB coupled with the inability to utilize β -hydroxybutyrate	+	+
Production of 2,3-butanediol	+	+
Hydrolysis of:		
Starch	—	—
Chitin	+	+
Alginate	—	—
Gelatin	—	—
Lipase	—	—
Luminescence	+	—
Sensitivity to O/129	+	+
Na ⁺ required for growth	+	+
Growth in NaCl:		
0%	—	—
2%	+	+
5%	+	+
7%	—	—
Growth at:		
4°C	+	+
20°C	+	+
30°C	(1)	(1)
35°C	—	—
Utilization of:		
D-ribose, D-fructose, D-glucose, D-mannose, D-galactose, Maltose, glycerol	+	+
D-gluconate, D-glucuronate	+	—
Pyruvate	(1)	(1)
DL-glycerate	+	(1)
Succinate, fumarate	(2)	(1)
L-glutamate	(1)	+
L-serine	(2)	(2)
Acetate, trehalose, L-proline, Valerate, DL-malate, citrate, D-xylose, L-arabinose, L-rhamnose, lactose, salicine, sucrose, D-mannitol, D-sorbitol, ethanol, n-propanol, β -alanine, L-leucine, L-citrulline, L-histidine, L-ornithine, betaine, cellobiose, melibiose	—	—
Mol% G+C in DNA	41–42	40–43

^a Strain N-XBO, N-KGF and N-VEF.^b Strain N-YAG, N-MHL and N-611A.

+, all positive; —, all negative; (), No. of positive strains.

micrographs of negatively stained cells of the test strains showed that each flagellum was about 15 nm thick and was not enclosed in a sheath. The colonies of the strains were convex, smooth with an entire edge and white in color on a complex medium. All of the strains grew well at 4°C, but did not grow at 35°C. They required Na⁺ for growth and grew well in a medium containing 2% NaCl.

Table 2. Levels of DNA-DNA relatedness between the histamine-producing strains and three species in the genus *Photobacterium*

Source of unlabeled DNA	% Relatedness to labeled DNA from	
	N-XBO	N-YAG
Histamine-producing strains		
N-XBO	100	NT
N-YAG	NT	100
<i>Photobacterium phosphoreum</i> ATCC 11040 ^T	100	88
<i>Photobacterium leiognathi</i> ATCC 25521 ^T	52	66
<i>Photobacterium angustum</i> ATCC 25915 ^T	47	54
<i>Photobacterium histaminum</i> JCM 8968 ^T	37	53

NT, not tested.

They could grow neither in 0% nor 7% NaCl broth. All of the strains fermentatively decomposed D-glucose to gas and other acidic end products. They accumulated poly- β -hydroxybutyrate in the cells, but did not utilize exogenous monomer β -hydroxybutyrate. Acetoin and 2,3-butanediol were produced from glucose. Lipase and gelatinase were not produced. The strains examined gave different reactions to the oxidase test. Strains N-YAG, N-MHL and N-611A did not show luminescence. All of the strains grew in the mineral medium containing D-glucose, but not in the medium containing D-mannitol.

The DNA base composition was found to be 40 to 43 mol% G+C for the strains. The results of DNA-DNA hybridization with labeled DNAs from strains N-XBO and N-YAG are summarized in Table 2. *P. phosphoreum* ATCC 11040^T exhibited high levels of homology with strain N-XBO and with strain N-YAG, whereas the levels of DNA homology between these strains and *P. leiognathi* ATCC 25521^T, *P. angustum* ATCC 25915^T and *P. histaminum* JCM 8968^T were lower, less than 70%.

Discussion

The strains which we examined were found to represent a very homogenous group of bacteria and thought to belong to a single species on the basis of the phenotypic characteristics. They were Gram-negative coccobacilli, motile by means of unsheathed polar flagella, endospore-negative, facultatively anaerobic, positive for D-glucose fermentation and poly- β -hydroxybutyrate accumulation. Also, the G+C contents of DNA (mol%) of them were 40 to 43. It is considered that the N-group bacteria belong to genus *Photobacterium* in comparison with the properties of this genus described so far.

The genus *Photobacterium* now consists of five validly described species, *P. phosphoreum*, *P. leiognathi*, *P. angustum*, *P. damsela* and *P. histaminum*. The former three species were listed on the Bergey's Manual of Systematic

Table 3. Characteristics for differentiating five species of genus *Photobacterium*

Species	Gas from D-glucose	Bioluminescence	Lipase	Gelatinase	Utilization of						Growth at	
					D-Xylose	Maltose	Acetate	DL-Glycerate	Pyruvate	L-proline	4°C	35°C
The N-group bacteria												
3 luminous strains	+	+	—	—	—	+	—	+	(1)	—	+	—
3 nonluminous strains	+	—	—	—	—	+	—	(1)	(1)	—	+	—
<i>P. phosphoreum</i> * ¹	+	+	—	—	—	+	—	+	—	—	+	—
<i>P. leiognathi</i> * ¹	—	+	+	—	—	—	+	D	+	+	—	+
<i>P. angustum</i> * ¹	—	—	D	D	+	D	+	—	+	—	D	+
<i>P. damsela</i> * ²	D	—	+	—	—	ND	—	+	+	ND	—	+
<i>P. histaminum</i> * ³	+	—	ND	—	—	+	+	+	—	+	—	+

+, positive; —, negative; (), no. of positive strains; D, positive or negative; ND, no data.

*¹ Data from Bergey's Manual of Systematic Bacteriology.¹⁵⁾

*² Data from Smith *et al.*¹⁶⁾

*³ Data from Okuzumi *et al.*³⁾

Bacteriology¹⁵⁾ in 1984, and under current taxonomic status another two species were added into genus *Photobacterium*.

Table 3 indicates characteristics for differentiating *Photobacterium* species quoted from the previous literatures.^{3,15-17)} The following combination of properties distinguishes *P. phosphoreum* from former species, *P. leiognathi* and *P. angustum*: production of gas from D-glucose; no growth at 35°C and no assimilation of D-xylose, acetate, pyruvate and L-proline as the carbon source. Moreover, *P. phosphoreum* is positive for growth at 4°C, luminescence and utilization of maltose and DL-glycerate and negative for production of gelatinase and lipase.¹⁵⁾ Therefore, the six test strains are almost identical to *P. phosphoreum* in the phenotypic characteristics.

Recently two species of *P. damsela*¹⁶⁾ and *P. histaminum*³⁾ were added into genus *Photobacterium*. *Listonella damsela* (initially described as *Vibrio damsela*) was isolated from skin lesions on damselfish, and transferred into the genus *Photobacterium* as *P. damsela*. *P. histaminum* was histamine-producing marine bacteria. The N-group bacteria differed from two new *Photobacterium* species in growth temperatures. Six test strains of the N-group bacteria grew well at 4°C, even at 0°C. But *P. damsela* could not grow at 4°C and grew much better at 25°C than 36°C. *P. histaminum* was mesophiles which could not grow at 4°C, and grew much better at 30°C and 35°C.

Slight differences were apparent between the N-group bacteria and *P. phosphoreum*. Different results of oxidase has been often obtained with the isolates of the N-group bacteria.⁴⁾ Most of *P. phosphoreum* strain is oxidase negative (10.1% to 25% positive¹⁷⁾), but Reichelt and Baumann¹⁸⁾ showed that some strains of *P. phosphoreum* gave positive results when treated with toluene prior to the addition of the oxidase reagent. Therefore, it may appear that the diagnostic trait circumscribing for the genus *Photobacterium* does not include oxidase test.

There was variability in the ability to luminesce between the N-group bacteria.⁴⁾ Strains N-XBO, N-KGF and N-VEF were able to emit light of blue-green color, whereas strains N-YAG, N-MHL and N-611A do not. The ability to luminesce appears to be restricted to the species *P. phosphoreum* and *P. leiognathi* among members of the genus *Photobacterium*.¹⁵⁾ Therefore this character of latter three strains of the N-group bacteria cannot coincide with that

of *P. phosphoreum*. However, according to Baumann *et al.*¹⁵⁾ strains of all luminous species develop dark mutants (nonluminous mutants) and may predominate under certain conditions. These facts suggest that nonluminous strains of the N-group bacteria are nonluminous mutants of *P. phosphoreum*.

DNA-DNA hybridization data verify the taxonomic position of two strains, N-XBO (luminous strain) and N-YAG (nonluminous strain) as being members of *P. phosphoreum*, and the high levels of homological similarity between the isolates and *P. phosphoreum* were observed. Consequently, they are phylogenetically closely related and it is considered that the N-group bacteria fall into a single taxon and seem to be members of the species *P. phosphoreum*.

Besides our previous literatures on histamine-producing luminous bacteria,⁴⁻⁸⁾ Morii *et al.* reported that luminous bacteria produced histamine when scombroid fish was stored at low temperatures and these isolates belonged to *P. phosphoreum* on the basis of their phenotypical traits.^{19,20)} Nonluminous isolates of the N-group bacteria were also identified as *P. phosphoreum* in this study. This fact indicated that *P. phosphoreum* should receive increased attention as histamine-producing marine bacteria.

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