On Two New Species of *Spirillum*

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The present author reported on the isolation and the taxonomic studies of a new species of *Spirillum*, *Spirillum putridiconchylum*¹⁾. Thereafter he isolated new species of *Spirillum* from the putrid bodies of two species of the fresh water shell fishes, *Corbicula japonica* Prime and *Cipangopaludina malleata* (Reeve). This paper deals with the isolation and the morphological, cultural and physiological studies of these spirilla.

Isolation

Source of materials: *Corbicula japonica* Prime used in this study was purchased at the market at Takanobashi in Hiroshima city in October 1958; *Cipangopaludina malleata* (Reeve) was collected at the paddy-field at Kairosiohama in Itsukaichi-cho, near Hiroshima city in May 1959. The putrid media in which the spirilla had developed were obtained by the same procedure as mentioned in a previous paper¹⁾. Among several organisms which were isolated by plating on the putrid media, the following two spirilla were identified as the new species *Spirillum metamorphum* nov. sp. (isolated from the putrid medium of *Corbicula japonica* Prime) and *Spirillum crassum* nov. sp. (from the putrid medium of *Cipangopaludina malleata* Reeve).

Method and Media: Method for the isolation is the same as in *Spirillum putridiconchylum*¹⁾. But two media employed in this study were as follows. a) For *Spirillum metamorphum*; peptone 5 g., yeast extract 3 g., sodium chloride 1 g., shell fish extract 200 ml., water 800 ml. Preparation of the liquid media and the solid media is the same as that mentioned previously¹⁾ except the sort of shell fish used to obtain the extract, to say, in this case *Corbicula japonica* Prime was used. b) For *Spirillum crassum*; peptone 5 g., beef extract 3 g., yeast extract 3 g., water 1000 ml. For preparing the solid media, 0.7% (w/v), agar powder was added. The pH of media was adjusted to 7.0 to 7.2 before sterilizing.

Culture media and cultural conditions for the identification of the spirilla

Culture media and cultural methods for the identification of the spirilla were the same as used for the identification of *Spirillum putridiconchylum*¹⁾, but the pH of the culture media and the temperature of incubation were as follows. The pH of the media was adjusted to 8.0 to 8.2 for *Spirillum metamorphum*, and 7.4 to 7.6 for *Spirillum crassum*; the temperature of incubation was 30° for *Spirillum metamorphum*, and 38 to 40° for *Spirillum crassum*. The gelatine plate and stab were incubated at 20° for the both species.

Descriptions of the species *Spirillum metamorphum* nov. sp.

1. Morphological characteristics

Vegetative cell: The initial cultures of the spirillum contain the definite spiral

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cells in high percentage, but the repeated subcultures scarcely contain the definite spiral cells. The spirillum grown in the nutrient broth after incubation for 24 hrs. has a rod- or a vibrio-shaped cell which is 2 to 12 microns in length, or a S- or a spiral-shaped cell which is 10 to 20 microns in length, with obtuse ends. In the spiral-shaped cell, the wave length is 10 to 12 microns and the width of the wave is 2 to 3 microns. The S- or the spiral-shaped cell often appears to be composed of two or more cells. The diameter of the cell is 1.2 to 1.4 microns. Refractive granules are seen in some individuals but not in others. The motility of the spirillum is screwlike in the rod- or the vibrio-shaped cell as well as in the S- or the spiral-shaped cell. In old culture, the vegetative cells predominate and the microcysts are not found. The cell taken from a colony on the nutrient agar plate after 24 hrs. is a rod- or a vibrio-shaped cell of 2 to 6 microns in length. The diameter of the cell is almost the same as that of the cell grown in the nutrient broth. Concerning the refractive granules, no differences are observed between the cell grown on agar and that grown in broth. The spirilla begin to move as soon as they are transferred into the liquid media.

Stained cell: The spirillum which is grown in the nutrient broth and the spirillum which is obtained from the colony on agar exhibit almost the same appearances. When the cell of 24 hrs. old is stained with Löffler's methylene blue, it shows somewhat different appearances, such as (1) the feature which is uniformly colored heavy blue without showing any structure, (2) the feature of several small volutin granules, taking purplish-red color, scattered in the body which is uniformly dyed blue or which reveals the so-called reticulate appearance. The volutin granules are most abundantly found in the cell after incubation for 2 days. The flagella are readily stained by Löffler's method. The young cells of the spirillum possess single flagellum or a tuft of 2 to 3 flagella at both poles of the body. The number of flagella increases as the culture becomes older. After 2 days or more, the cell possesses a tuft of several flagella at each pole. The spirillum is Gram-negative.

2. Cultural characteristics

Agar colonies: Growth is speedy. Colonies are seen with the naked eye after 24 hrs. After 48 hrs., surface colonies are circular or slightly irregular (1.0 to 1.4 mm. in diameter) with smooth surface and small indented margin, and are finely granular, convex, creamy-white, opalescent, and brittle. Deep colonies are smaller and round, elliptical, spindle or irregular.

Gelatine colonies: Colonies are seen with the naked eye after 48 hrs. After 5 days, surface colonies are punctiform or circular (0.9 to 1.1 mm. in diameter) with rough surface, and are lacerate, coarsely granular, creamy-white, opaque, and brittle. Gelatine plate is liquefied, forming a slightly concave surface. Deep colonies are the same as the surface colonies except that they are smaller, and that they have a small indented margin.

Agar stroke: Growth is moderate, beaded, creamy-white, glistening, and brittle. Fetid odor is absent, and the medium is unchanged.

Agar stab: Growth is seen along the entire stab line and on the surface, but scant in the lower part of the stab. The line of puncture is papillate, and the medium is unchanged.

Gelatine stab: Growth is seen along the entire stab line, but scant in the lower part of the line. Liquefaction begins in 5 days. Form of liquefaction is napiform for about 10 days, but becomes infundibular as the culture becomes older. After 4

weeks, liquefaction is about 25 mm. in depth.

Nutrient broth: Growth is moderate. After 24 hrs., thin membranous masses develop on the surface of medium along the tube wall. The masses precipitate by slight agitation. A thin ring remains along the tube wall after the masses have precipitated, and it disappears in about 5 days and finally the medium becomes clear. Fetid odor is absent.

3. Physiological characteristic

Growth appears on the surface and in upper ca. 3 mm. of an agar shake cultured,

A. *Spirillum metamorphum* nov. sp.

Figs. 1-16. Fig. 1, vegetative cells in initial culture, showing the definite spiral form. $\times 1200$. Fig. 2, vegetative cells in the nutrient broth after 24 hrs. at 30° , showing the vibrio- or the S-shaped form. $\times 1200$. Fig. 3, vegetative cells obtained from a colony on the nutrient agar plate after 24 hrs. at 30° , showing the short rod- or the vibrio-shaped form. $\times 1200$. Figs. 4 and 5, specimens stained with Löffler's methylene blue. $\times 1200$. The cells in Fig. 4 are obtained from a colony on the nutrient agar plate after 24 hrs. at 30° . The cells in Fig. 5 after 48 hrs. at 30° , containing the small volutin granules. Fig. 6, flagella-staining by Löffler's method. The cells are obtained from a colony on the nutrient agar plate after 24 hrs. at 30° . $\times 1200$. Fig. 7, a surface colony on the nutrient agar plate after 48 hrs. at 30° . $\times 15$. Fig. 8, the margin of the same colony as in Fig. 7. $\times 50$. Figs. 9 to 12, deep colonies on the nutrient agar plate after 48 hrs. at 20° . $\times 15$. Fig. 13, a surface colony on the nutrient gelatine plate after 5 days at 20° . $\times 15$. Fig. 14, the margin of the same colony as in Fig. 13. $\times 50$. Fig. 15, a deep colony in the nutrient gelatine plate after 5 days at 20° . $\times 15$. Fig. 16, the margin of the same colony as in Fig. 15. $\times 50$.

but not in the lower part. The spirillum does not reduce nitrate to nitrite, and produces neither indole nor hydrogen sulfide. Catalase is not produced by growth on the nutrient agar. The spirillum produces neither acid nor gas from glucose, fructose, lactose, sucrose and mannitol. Litmus milk is not coagulated, and shows a slight alkaline-reaction in ca. 1 week, but it is decolorized at bottom. This appearance does not change later. The optimum temperature for growth is 30°. The spirillum can grow at 37°, but not above 40°. The optimum pH is 7.6 to 8.4, and growth can be initiated between pH 6.0 and 9.6. Methyl red and Voges-Proskauer reaction are negative. The spirillum does not grow in synthetic media containing any of succinate, fumarate, malate, lactate, acetate, propionate, butyrate, citrate, malonate, glucose, fructose, glycerol and ethyl alcohol as a sole carbon source, and any of ammonium salts, nitrate, urea and asparagine as a sole nitrogen source.

Spirillum crassum nov. sp.

1. Morphological characteristics

Vegetative cell: The spirillum grown in the nutrient broth after incubation for 24 hrs. has a stout cell, with obtuse ends. It is generally 10 to 40 microns in length, but occasionally the cell reaches the length of 100 microns. The diameter of the cell is 1.3 to 1.5 microns. The waves are shallow, regular and 1 to 6 in number. The wave length is 6 to 7 microns. The width of the wave 1.3 to 1.5 microns. Refractive granules are present in some individuals but not in others. The spirillum moves to either direction with equal rapidity and facility, rotating counterclockwise. In old cultures, the vegetative cells predominate and the microcysts are not found. The cell taken from a colony on the nutrient agar plate of 24 hrs. old is shorter than that taken from the nutrient broth. A great number of the cells show the vibrio- or the short spiral-shaped form. The cell is generally 3 to 15 microns in length, but occasionally reaches the length of 20 microns or more. The diameter of the cell is almost the same as that of the cell grown in the nutrient broth. Concerning the refractive granules, no differences are observed between the cells grown on agar and that grown in broth. The spirilla begin to move as soon as they are transferred into the liquid media.

Stained cell: The spirillum cultivated in the nutrient broth and the spirillum taken from the colony on agar plate exhibit almost the same staining features. When the spirillum incubated for 24 hrs. is stained with Löffler's methylene blue, it shows the cell which is uniformly colored light or heavy blue, including several volutin granules. The reticulate structure is not clear. The volutin granules are most abundantly found in the cell of one to two days old cultures. The flagella are readily stained by Löffler's method. Most of the spirillum after 24 hrs. have a tuft of several to about ten or more flagella at both poles of the cell. The spirillum grown in the nutrient broth has as many flagella as the spirillum obtained from a colony on agar. The spirillum is Gram-negative.

2. Cultural characteristics

Agar colonies: Growth is speedy. After incubation for 24 hrs., surface colonies are circular or slightly irregular (1.0 to 1.5 mm. in diameter) with smooth surface, and are coarsely granular, convex, light brown, opalescent, and brittle. The outer part of the colony appears as a thin transparent layer, and the inner part is granular and has lacerate margin. The outer layer disappears after ca. 1 day. Deep colonies

are smaller than the surface ones, and are round, elliptical or irregular. Their margin are lacerate or lobate. As the culture becomes older, the surface colonies become larger and show a change in margin and in elevation. Namely, after 5 days the diameter of the colonies is 4.0 to 5.0 mm., the margin is undulate, and the elevation is umbonate. Deep colonies show the same characteristics as after 24 hrs. except that the diameter is 1.5 to 2.0 mm. and that the margin is lacerate in all colonies.

Gelatine colonies: Colonies are seen with the naked eye after 2 days. After 5 days, surface colonies are circular (1.0 to 1.6 mm. in diameter) with rough surface, and are coarsely opaque, flatt, brown, opaque, and brittle. Concerning the margin, there are seen the following three different types: the first is curled, the second lacerate, and the third ciliate. Deep colonies are punctiform (ca. 0.4 mm. in diameter), and have the lacerate margin. As the culture becomes older, the margin of the curled margin-colonies changes into lacerate or ciliate. After 1 week the surface colonies have the lacerate or ciliate margin, and show a concentric stratification with a filamentous structure. Gelatine plate is liquefied, forming a slightly concave surface. Deep colonies are filamentous (0.8 to 1.2 mm. in diameter), and brown.

Agar stroke: Growth is beaded, creamy-white, glistening, and brittle. Fetid odor is absent, and the medium is unchanged.

Agar stab: Growth is seen along the entire stab line, and on the surface. The line of puncture is papillate in ca. 1 week, but afterwards changes to beaded. The medium is unchanged.

Gelatine stab: Growth is seen along the entire stab, but is scanty in the lower part. Growth on the surface develops fairly around the point of puncture. The line of puncture is beaded, and the gelatine is slowly liquefied in crateriform. After 4 weeks, liquefaction is ca. 8 mm. in depth.

Nutrient broth: Growth is speedy. After 24 hrs., a thin membrane develops on the surface. The medium is densely turbid. The membrane sinks into the bottom in a lump by slight agitation. After the membrane has sunk, the ring remains along the tube wall. The turbidity of the medium becomes light as the culture becomes older, but it still remains 2 weeks later. The ring still remains at that time. Fetid odor is absent.

Potato: No growth occurs.

3. Physiological characteristics

The spirillum grows on the surface and in upper ca. 3 mm. of an agar shake culture. It does not reduce nitrate, and does not produce indole and hydrogen sulfide. Catalase is not produced by growth on the nutrient agar. The spirillum produces neither acid nor gas from glucose, fructose, sucrose, lactose and mannitol. Litmus milk is unchanged. The optimum temperature for growth is 38 to 40°. The organism can grow at 45°, but not at above 48°. The spirillum grows well from the pH 6.5 to 8.5, and growth can be initiated between pH 5.8 and 9.4. Methyl red and Voges-Proskauer reaction are negative. The spirillum utilizes asparagine well and urea slightly as sole nitrogen sources in synthetic media, but not the salts of ammonium and nitrate. The spirillum utilizes the salts of succinic, fumaric, pyruvic, malic, and lactic acid, glucose and glycerol as sole carbon sources in synthetic media, but not the salts of acetic, propionic, butyric, citric, and malonic acid, fructose and ethyl alcohol.

B. *Spirillum crassum* nov. sp.

Figs. 1-17. Fig. vegetative cells moving slowly in the nutrient broth after 24 hrs. at 39°, photographed with a dark contrast phase microscope. $\times 1200$. Figs. 2 and 3, specimens stained with Löffler's methylen blue. $\times 1200$. The cells in Fig. 2 are obtained from a colony grown on the nutrient agar plate after 24 hrs. at 30°. The cells in Fig. 3 after 72 hrs. Fig. 4, flagella-staining by Löffler's methods. The cells are obtained from a colony grown on the nutrient agar plate after 24 hrs. at 39°. $\times 1200$. Fig. 5, a surface colony on the nutrient agar plate after 24 hrs. at 39°. $\times 15$. Fig. 6, the margin of the same colony as in Fig. 5. $\times 50$. Fig. 7, the margin of the colony after 5 days. $\times 15$. Fig. 8, a deep colony on the nutrient agar plate after 24 hrs. at 39°. $\times 15$. Fig. 9, the margin of the same colony as Fig. 8. $\times 50$. Figs. 10, 12 and 14, surface colonies on the nutrient gelatine plate after 5 days at 20°. $\times 15$. Figs. 11, 13 and 15, each margin of the same colonies as in Figs. 10, 12 and 14. $\times 50$. Fig. 16, a deep colony on the nutrient gelatine plate after 5 days at 20°. $\times 15$. Fig. 17, the margin of the same colony as in Fig. 16. $\times 50$.

Discussion

Spirillum metamorphum is thicker than 1 micron in the diameter of the cell. Although the initial culture of the spirillum contains the definite spiral cells in high percentage, the subcultures scarcely contain the definite spiral forms. Even when the spirillum shows the spiral form, it is nothing but a chain of cells which is caused

by successive cell division. As *Spirillum* which possesses such morphological characteristics, the following two species have been reported: *Spirillum kutscheri* Migula^{2,3,4}) and *Spirillum giesbergeri* Williams et Rittenberg⁵). The spirillum isolated by the present author can not be distinguished morphologically from those species because it is markedly similar to them also in the other morphological properties. The spirillum in question, however, differs from them in some cultural and physiological characteristics. The differences among these spirilla are expressed in Table 1.

Table 1. The differences among *Spirillum kutscheri*, *Spirillum giesbergeri*, and *Spirillum metamorphum* nov. sp. isolated by the present author.

	<i>Spirillum kutscheri</i> Migula	<i>Spirillum giesbergeri</i> Williams et Rittenberg	<i>Spirillum metamorphum</i> nov. sp.
Agar colony	deep; dark brown; round or whetstone-form	surface: white; lobate	surface: creamy-white; small indented margin
Agar stab	filiform		deep: creamy-whith; round, elliptical or spindle
Gelatine colony	surface: transparent; whetstone-form or round; lobate		papillate
Gelatine stab	slow liquefaction*	no liquefaction	surface: creamy-white; punctiform or circular; lacerate
Potato	limited growth*	very slight, white growth	liquefaction; napiform in 10 days, but infundi- bular afterwards
Catalase	positive*	positive	no growth
Optimum temperature	22-27°		negative
Available nitrogen compound	ammonium salts*	ammonium salts, nitrate, urea, and asparagine	30-32°
Available carbon compound	salts of malic, and pyruvic acid*	salts of pyruvic, malic, and lactic acid	no growth in synthetic media
	salts of malic, and succinic acid**		

* Descriptions according to Giesberger's monograph⁶).

** Descriptions according to Bergy's Manual.

As shown in Table 1, the spirillum isolated by the present author differs from *Spirillum kutscheri* and *Spirillum giesbergeri* in eight important respects for the identification. The most striking characteristics by which the spirillum in question is distinguished from the above mentioned two species are its inability to grow on potato, the absence of catalase, and its inability to grow in synthetic media. From the above-mentioned evidence, the spirillum isolated from *Corbicula japonica* Prime is diagnosed as a new species, and it is named *Spirillum metamorphum* from its metamorphosis.

Spirillum crassum isolated from *Cipangopaludina malleata* (Reeve) distinctly differs from all definite species^{4,5,6}) described up to the present time in several important characteristics. Especially the following characteristics are sufficient to diagnose the spirillum as a new species. Namely, (1) the spirillum has a stout cell of 1.3 to 1.5

microns in diameter; (2) the optimum temperature for growth is 38 to 40°, at which most of species can not grow; (3) the spirillum is catalase-negative; (4) the spirillum can utilize asparagine well, but urea is less readily utilized; the salts of ammonium and nitrate are not utilized as a sole source of nitrogen for growth; (5) the spirillum can utilize the salts of succinic, fumaric, pyruvic, lactic, and malic acid, glucose and glycerol as sole carbon sources, but not utilize the salts of acetic, propionic, butyric, citric, and malonic acid, fructose and ethyl alcohol. From the above points of view, the spirillum isolated from *Cipangopaludina malleata* (Reeve) is diagnosed as a new species, and it is named *Spirillum crassum* because it has a stout cell.

Summary

Two new species of *Spirillum* have been isolated from the putrid media in which the putrid bodies of two species of the fresh water shell fishes, *Corbicula japonica* Prime and *Cipangopaludina malleata* (Reeve) laid. On the basis of the morphological, cultural and physiological characteristics, the spirillum isolated from *Corbicula japonica* Prime is diagnosed as a new species, and it is named *Spirillum metamorphum* from its metamorphosis; and also the spirillum isolated from *Cipangopaludina malleata* (Reeve) is diagnosed as a new species and named *Spirillum crassum* because it has a stout cell.

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摘 要

寺崎弥助: *Spirillum* 二新種について

1958年10月、広島市鷹の橋マーケットで購入したヤマトシジミ、および、1959年5月、広島郊外五日市町海老塩浜の稲田で採集したマルタニシの腐敗液から、寒天平板塗抹法により *Spirillum* をそれぞれ一つずつ分離した。分離に用いた培地成分は、前者では、ペプトン、酵母エキス、ヤマトシジミせんじ汁、食塩であり、後者では、肉エキス、ペプトン、酵母エキスである。両者を培養し、形態学的、培養的、生理学的性質をしらべ、正確な種として記載された *Spirillum* と比較検討した結果、それらを新種と断定した。ヤマトシジミより分離した *Spirillum* は、分離当初においては明瞭ならせん形を示したが、継代培養数回後には体長の短い棒状あるいは *Vibrio* 状に変形し、現在も同様な形態を示している。まれにらせん形の個体がみられるが、二個あるいはそれ以上にくびれている場合が多い。この形態変化により、この菌を *Spirillum metamorphum* と命名した。マルタニシより分離した *Spirillum* は菌体が太く強固であることにより、*Spirillum crassum* と命名した。(鈴峰女子短期大学生物学教室)