Rhodococcus marinonascens sp. nov., an Actinomycete from the Sea

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Two groups of nocardioform organisms isolated from marine sediments were studied, along with representative strains of the genera *Rhodococcus*, *Nocardia*, and *Mycobacterium*. Strains of the red- or orange-colored group showed no marine affinity and exhibited a close relationship to *Rhodococcus rhodochromus*. The 17 marine isolates of the cream-colored group formed a homogeneous well-separated cluster and were particularly characterized as moderately halophilic and psychrotrophic organisms. These isolates were assigned to a new species within the genus *Rhodococcus*. We propose the name *Rhodococcus marinonascens*, referring to the exclusive occurrence of this taxon in the sea, and describe the properties of the species and of type strain 3438W (= DSM 43752).

In the bottom sediments of various open sea sites in the northeastern Atlantic Ocean (North Atlantic Ridge, Iberian Sea, Bay of Biscay, North Sea, off the Faeroe Islands, Norwegian Sea, and Barents Sea), nocardioform bacteria have been detected (34, 35). The marine nocardioform isolates can be divided into two groups on the basis of pigmentation type. The majority of the isolates are cream colored, moderately halophilic, and psychrotrophic, whereas the other isolates are red or orange and obviously not adapted to the conditions of the marine environment. Preliminary morphological and physiological studies have shown clear differences between the red or orange organisms and the cream-colored organisms, but within a color group the strains are almost identical. To define the taxonomic status of the marine strains, we used chemotaxonomic and numerical methods. The marine nocardioform isolates were compared with representatives of the genera *Mycobacterium*, *Nocardia*, and *Rhodococcus* and with strains of organisms not included on the Approved Lists of Bacterial Names (30). The marker strains were selected partially on the basis of previous chemotaxonomic studies which indicated some relationship to the marine organisms.

MATERIALS AND METHODS

Organisms. The strains included in this taxonomic study are listed in Table 1. The reference strains were received from the Deutsche Sammlung für Mikroorganismen, Göttingen, Federal Republic of Germany. The marine strains were isolated from the uppermost layer of marine sediments from various sea sites by using methods and media previously described (35, 36).

Test conditions. For purposes of comparison, the cultural and physiological tests were carried out with all strains under the same conditions. Unless stated otherwise, we used an incubation temperature of 18°C and test media containing 75% seawater or an equivalent salt content. The cultures used for inoculation of test media were grown in liquid yeast extract-malt extract medium (36) for 8 days and then centrifuged, washed with tap water, suspended in liquid BMS medium (see below), and homogenized for 5 s by using a high-speed blender (Ultraturrax; IKA Werk, Staufen, Federal Republic of Germany).

The growth and utilization tests were performed in polystyrene replidishes that were divided into 25 separate compartments (Greiner, Nürtingen, Federal Republic of Germany). The dishes were inoculated with a multipoint inoculator (Greiner). Degradation of different substrates and susceptibility to antibiotics were studied in plastic petri dishes.

Nitrate reduction, resistance to lysozyme, and urease activity were examined in test tubes.

Physiological tests. The production of acid from different carbohydrates was detected in BMS-N medium, which contained 1.0 g of $(\text{NH}_4)_2\text{HPO}_4$, 0.2 g of KCl, 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 25.0 g of NaCl, 15.0 g of agar no. 1 (Oxoid Ltd., London, England), 1,000 ml of distilled water, and 15 ml of a 0.04% (wt/vol) aqueous bromocresol purple solution. The pH of this medium was adjusted to 7.0 with 1 N HCl. After autoclaving, the carbohydrate to be tested (final concentration, 1% wt/vol) and yeast nitrogen base without amino acids (final concentration, 0.067%, wt/vol; Difco Laboratories, Detroit, Mich.) were added aseptically. Most of the carbohydrates were sterilized by filtration. Inulin, dextrin, and salicin were autoclaved separately, and dulcitol was sterilized by steaming for 30 min on 3 consecutive days. The inoculated media were scored for changes in color (indicating acid production) every week for 5 weeks. The last reading was taken after 2 months.

The tests for utilization of organic compounds as sole carbon sources were done with BMS-N medium without bromocresol purple. The different carbon sources were added to give final concentrations of 0.1% (wt/vol).

Tyrosine, DL-norleucine, testosterone, *p*-hydroxybenzoic acid, and sebacic acid were autoclaved; the remaining organic compounds were sterilized by filtration.

Growth on the inoculated media was recorded after 8, 16, 30, and 48 days. The last scoring was after 3 months. The test was considered positive if growth in the test dish was greater than growth in the control dish without any carbon source.

The tests for utilization of organic compounds as sole carbon and nitrogen sources were carried out in basal medium BMS, which contained 0.5 g of KH_2PO_4 , 1.5 g of $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.2 g of KCl, 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 25 g of NaCl, 15.0 g of agar no. 1 (Oxoid), and 1,000 ml of distilled water (pH 7.0). The amino acids L-serine, L- α -alanine, and glucosamine were sterilized by filtration and were added to the sterile medium to give final concentrations of 0.1% (wt/vol).

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TABLE 1. List of strains used in this study

Cluster	Serial no.	Name or laboratory no.	Source or location of sediment sample	Depth of sediment sample (m)
1	1	2808W	25°36'N, 16°07'W	940
	2	1801W	36°47'N, 14°16'W	408
	3	2708W	46°58'N, 05°13'W	160
	4	3228W	80°10'N, 11°10'E	197
	5	4578W	Jacotet Bay, Mauritius	5
	6	3438W ^T	51°30'N, 30°19'W	1,400
	7	7579W	52°16'N, 31°00'W	700
	8	4169W	76°18'N, 11°32'E	2,800
	9	7584W	52°16'N, 31°00'W	700
	10	4140W	53°18'N, 34°39'W	1,680
	11	5216W	Bethy's Bay, Republic of South Africa	12
	12	2834W	33°40'N, 09°09'W	1,750
	13	4412W	78°26'N, 07°06'E	3,000
	14	4251W	75°36'N, 13°29'E	1,025
	15	3609W	70°20'N, 15°24'E	2,342
	16	4170W	76°49'N, 12°01'E	1,208
	17	2863W	47°18'N, 06°32'W	1,100
?	18	5075W	Coastal soil, Seychelles	
?	19	4599W	Coastal soil, Mauritius	
2	20	<i>Mycobacterium smegmatis</i>	DSM 43078 ^a	
	21	<i>Mycobacterium phlei</i>	DSM 43214	
?	22	<i>Nocardia asteroides</i>	DSM 43003	
3	23	<i>Rhodococcus</i> sp. (Hüls AG, Marl; " <i>Nocardia corallina</i> ")	DSM 43001	
	24	<i>R. rhodochrous</i> (K. Schaal N27; " <i>Nocardia rubra</i> ")	DSM 43008	
	25	<i>R. rhodochrous</i>	DSM 43241 ^T	
	26	1732R	34°01'N, 15°19'W	3,920
	27	666R	51°26'N, 02°28'E	34
	28	3609R	70°20'N, 15°24'E	2,342
	29	4107R	52°24'N, 31°30'W	1,080
	30	2863R	47°18'N, 06°32'W	1,100
	31	4020R	79°18'N, 11°40'E	200
4	32	<i>R. erythropolis</i> Metcalf and Brown 1957 (= ATCC 19369); (" <i>Nocardia calcarea</i> ") ^b	DSM 43188	
	33	<i>R. erythropolis</i>	DSM 43066 ^T	
	34	<i>R. erythropolis</i> (L. Ettlinger LBG A3116; " <i>Nocardia opaca</i> ")	DSM 43135	
	35	<i>Nocardia</i> sp. (H. Reichenbach; " <i>Nocardia flava</i> ")	DSM 43069	
	36	<i>Nocardia</i> sp. (A. Seino KCC A0019; " <i>Nocardia polychromogenes</i> ")	DSM 43022	
?	37	<i>Nocardioides albus</i>	DSM 43109 ^T	

^a DSM, Deutsche Sammlung für Mikroorganismen, Göttingen, Federal Republic of Germany.

^b See reference 7.

Utilization was considered positive if growth in the medium containing the amino acid was greater than growth in the control medium without the amino acid. These tests were examined together with the tests for sole carbon sources.

Tests for hydrolysis of adenine (0.5%, wt/vol), guanine (0.5%, wt/vol), tyrosine (0.5%, wt/vol), keratin (0.5%, wt/vol), xanthine (0.4%, wt/vol), hypoxanthine (0.4%, wt/vol), and casein were performed by the method of Gordon et al. (15).

Hydrolysis of Tween 40, Tween 60, and Tween 80 was investigated by the method of Sierra (29).

Degradation of starch and chitin was examined by the method of Weyland et al. (37).

Cellulose degradation was examined in a medium containing 0.5 g of NaNO₃, 0.01 g of FeSO₄ · 7H₂O, 0.1 g of K₂HPO₄ · 3H₂O, 0.05 g of NaHCO₃, 0.25 g of (NH₄)₂SO₄, 0.05 g of yeast extract (Difco), 15.0 g of agar (Difco), 10.0 g of cellulose prepared by the method of Tansey (32), 750 ml of seawater, and 250 ml of distilled water (pH 7.0). The heavily inoculated plates were examined for clearing zones around or beneath the colonies.

Hydrolysis of cellulose, in the form of paper (Whatman no. 5) and cellophane (Kalle, Wiesbaden, Federal Republic

of Germany), and hydrolysis of esculin disks (Becton, Dickinson & Co., Rutherford, N.J.) and gelatin (charcoal gelatin disks; Oxoid) were examined in modified ZoBell medium (37). Hydrolysis was considered positive if the paper or cellophane was degraded, if the charcoal was released from the gelatin, or if the esculin disk colored the medium blackish brown.

All hydrolysis tests were recorded weekly for 5 weeks, with the final reading taken after 2 months. Starch degradation was analyzed after 5 weeks.

Catalase, oxidase, and urease were examined by the method of Weyland et al. (37). Formation of nitrate reductase was determined in yeast extract-malt extract medium containing 1.5 g of KNO₃ per liter. Formation of nitrite was detected with Griess-Ilosvay reagent after 3, 7, and 9 days.

Tests for tolerance to temperature, sodium chloride, pH and chemical inhibitors were carried out on PYA medium, which contained 10 g of Bacto-Peptone (Difco), 3 g of yeast extract (Difco), and 15 g of agar no. 1 (Oxoid). For the NaCl tests 1,000 ml of distilled water was added. For the remaining tests 750 ml of seawater and 250 ml of distilled water were used. In all tests except the pH tolerance tests the media were adjusted to pH 7.5.

We examined the ability of the organisms to grow at temperatures of 3, 12, 18, 24, and 30°C, at sodium chloride concentrations of 0, 1, 3, 4, 5, 7 and 10% (wt/vol), and in a pH range from 5 to 10.

In the inhibition tests, different concentrations (see Table 2) of crystal violet, sodium azide, potassium tellurite, phenyl ethanol, and phenol were added to PYA medium. A control culture without any inhibitor was used to estimate unaffected growth.

The results of the tolerance and inhibition tests were recorded after 1, 2, 4, and 8 weeks.

Resistance to lysozyme was determined by a modification of the method of Mordarska et al. (22). The incubation time after lysozyme (E. Merck AG, Darmstadt, Federal Republic of Germany) was added was extended to 6 h, and the incubation temperature was lowered to 24°C.

Susceptibility to penicillin was analyzed by using filter paper disks saturated with 2 and 10 IU of penicillin G (Difco). The disks were placed on yeast extract-malt extract agar inoculated with the different strains tested. The plates were observed for zones of growth inhibition around the disks after incubation periods of 3 and 10 days at 18°C. The tests for susceptibility to vancomycin and rifampin were carried out in the same manner, but the filter disks were prepared by using the method of Goodfellow and Orchard (13). The filter disks were soaked with a solution containing 50 µg of vancomycin (Serva) per ml or 50 µg of rifampin (Serva) per ml.

Morphological and cultural tests. Macroscopic growth was examined on yeast extract-malt extract agar plates, generally after incubation for 2 weeks (after good growth had occurred).

For microscopic observations, the slide culture technique of Williams and Cross (38) was used. Micrographs were obtained with a Zeiss Universal microscope, using phase- and interference-contrast equipment.

Chemotaxonomic tests. The amino acid, amino sugar, and monosaccharide components of cell wall fragments were determined by the method of Richter (27).

Mycolic acids were detected by performing a thin-layer chromatographic analysis of ethanol-diethyl ether extracts, using the method of Minnikin et al. (21).

Menaquinones were extracted and purified as described by Collins et al. (2). The purified menaquinones were examined by reverse-phase and silver ion-impregnated thin-layer chromatography (3, 16).

The guanine-plus-cytosine (G+C) contents of the deoxyribonucleic acids (DNAs) were calculated from the melting temperatures by using the formula of De Ley (4). The cells were cultivated and lysed by the method of Bourgeois and Beaman (1). The DNA was isolated by the method of Marmur (19). The melting temperature of the DNA was determined by the method of Marmur and Doty (20), using a Gilford model 250 spectrophotometer equipped with a Gilford model 2524 thermoprogrammer. DNA from *Escherichia coli* K-12 was used as a standard.

Computer analysis. The test organisms listed in Table 1 were screened for 106 unit characters. Final test readings were used for computation. A total of 22 characters gave either all positive or all negative results, and, as they had no discriminating value, these characters were omitted from the data matrix. A total of 84 unit characters (Table 2) were scored for computation by recording positive reactions as 2, negative reactions as 0, and weak positive reactions as 1.

The data were analyzed with a Nova model 1200 computer by using the Clust 6 program of H. Krause (Institut für

Meeresforschung Bremerhaven, Bremerhaven, Federal Republic of Germany). The subroutines necessary were obtained from Steinhausen and Langer (31). The between-strain similarities were calculated by using the euclidean distances. The cluster analysis was carried out by using the following eight methods: (i) single linkage; (ii) complete linkage; (iii) unweighted average linkage; (iv) weighted average linkage; (v) median method; (vi) centroid method; (vii) Wards method; and (viii) Lance-Williams method (17).

RESULTS

Numerical taxonomic analysis. Cluster analyses carried out with different methods (see above) produced rather similar results. Figure 1 shows the dendrogram derived by the unweighted average linkage cluster method. At the 80% similarity level there were four clusters, which contained 31 of the 37 strains tested.

(i) **Cluster 1.** Of the 19 cream-colored marine strains studied, 17 formed a homogeneous cluster with a relatively high intergroup similarity. This cluster did not contain any of the marker strains and was well separated from the other clusters. Therefore, it seems reasonable to equate cluster 1 with a new species.

Strain 5075W, one of the two cream-colored strains that were obtained not from the sea but from littoral sites, was only marginally included in cluster 1, and strain 4599W could not be allocated to any of the four clusters.

(ii) **Cluster 2.** Cluster 2 contained only the two reference strains of the genus *Mycobacterium*.

(iii) **Cluster 3.** The red or orange marine isolates formed a cluster together with the type strain of *Rhodococcus rhodochrous*, another strain (serial no. 24) of this species (previously labeled "*Nocardia rubra*"), and a *Rhodococcus* sp. strain (serial no. 23; formerly named "*Nocardia coralina*"). Four of the red or orange marine isolates showed a high affinity to the three marker strains, whereas strains 666R and 1732R were linked to the members of cluster 3 just above the 80% similarity level.

(iv) **Cluster 4.** The strains of *Rhodococcus erythropolis* were recovered in cluster 4. We found high affinity between the type strain (DSM 43066) and two other strains (DSM 43135 and DSM 43188) of this species (formerly labeled "*Nocardia opaca*" and "*Nocardia calcarea*," respectively). The latter designations have been reduced to synonyms of *R. erythropolis* by Goodfellow et al. (7). At a somewhat lower similarity level a *Nocardia* sp. strain (serial no. 35; "*Nocardia flava*") joined cluster 4.

The position of the marker strain *Nocardia* sp. (serial no. 36; "*Nocardia polychromogenes*") was dependent on the kind of cluster analysis method used. "*Nocardia polychromogenes*" was attached to the strains of cluster 4 or grouped together with *Nocardia asteroides*. In no case was the strain closely positioned to strains assigned to cluster 1.

Morphology and growth requirement. The strains of cluster 1 grew very slowly. Optimal growth occurred in media with a seawater content of 75 to 100% or with adequate salinity. On media prepared with distilled water no growth or only very scarce mycelia developed. The optimal growth temperature was about 20°C. Temperatures above 30°C inhibited growth almost completely, whereas fair growth occurred at 5°C after a relatively short incubation.

Under optimal growth conditions the strains formed well-developed branched primary mycelia (Fig. 2A through D), which fragmented after about 14 days into bacillary and coccoid elements (Fig. 3A through C). Aerial mycelia and diffusible pigments were not formed.

TABLE 2—Continued

Characteristic	No. of cluster 1 strains showing positive results (n = 17)	Result with type strain DSM 43752	Serial no. of cluster 1 strains showing negative results	No. of cluster 3 strains showing positive results (n = 9) ^b	Results with reference strains									
					R. erythropolis serial no. 34	R. erythropolis serial no. 33	R. erythropolis serial no. 32	Nocardia sp. serial no. 35	Nocardia sp. serial no. 36	Nocardia asteroides serial no. 22	M. smegmatis serial no. 20	M. phlei serial no. 21	Nocardioideis albus serial no. 37	
Sodium azide (0.2%, wt/vol)	17	+		1	-	-	-	+	-	+	+	+	+	
Sodium azide (0.3%, wt/vol)	17	+		2	-	-	-	+	-	+	+	+	+	
Potassium tellurite (0.01%, wt/vol)	4 (2)	(+)	1, 2, 3, 5, 10, 11, 12, 13, 14, 16, 17	0	-	(+)	-	-	-	(+)	-	-	-	
Potassium tellurite (0.05%, wt/vol)	8 (7)	(+)	10, 16	1 (1)	-	+	-	-	-	+	(+)	+	-	
Potassium tellurite (0.1%, wt/vol) ^d	11 (4)	(+)	10, 16	1 (1)	-	+	-	-	+	+	+	+	+	
Phenyl ethanol (0.3%, vol/vol) ^d	17	+		(4)	-	+	-	+	+	+	+	+	+	
Phenyl ethanol (0.4%, vol/vol)	17	+		3 (4)	+	+	-	+	+	+	+	+	+	
Phenol (0.01%, wt/vol) ^d	14 (2)	+	16	0	-	-	-	-	-	+	+	+	-	
Phenol (0.1%, wt/vol)	17	+		3 (3)	+	+	+	+	+	+	+	+	+	
Lysozyme ^d	2 (13)	(+)	10, 17	8 (1)	+	+	+	+	+	(+)	-	-	+	
Growth responses														
Growth with 7% NaCl	7 (4)	(+)	2, 3, 4, 7, 8, 10	1	+	+	+	+	+	+	(+)	(+)	-	
No or very weak growth without NaCl ^d	17	+		0	-	-	-	-	-	-	-	-	-	
Optimal growth with 3% NaCl ^d	17	+		0	-	-	-	-	-	-	-	-	-	
Optimal growth temp of about 20°C ^d	17	+		0	-	-	-	-	-	-	-	-	-	
No or very weak growth at 30°C ^d	17	+		0	-	-	-	-	-	-	-	-	-	
No or clearly reduced growth at pH 5 ^d	14 (3)	+		1 (4)	-	-	-	-	-	-	-	-	-	
No or clearly reduced growth at pH 10	5 (6)	(+)	5, 12, 14, 15, 16, 17	0	-	-	-	-	(+)	+	+	(+)	-	

^a The characteristics that gave identical results for all strains are not shown. The following characteristics were positive for all strains: propionate used as a sole carbon source; degradation of Tween 40, Tween 60, and Tween 80; growth with 1, 3, and 4% NaCl; and growth at pH 6 to 9. The following characteristics were negative for all strains: degradation of cellulose, cellophane, cellulose from paper, guanine, hypoxanthine, and keratin; formation of oxidase; and growth with pimelic acid, sebacic acid, *p*-cresol, and salicylaldehyde as sole carbon sources.

^b Including reference strains of *R. rhodochrous*.

^c +, Positive; -, negative; (+), weak positive.

^d Distinguishing characteristics for cluster 1 strains.

^e The numbers in parentheses are the numbers of strains that gave slight positive reactions.

FIG. 1. Dendrogram derived by the unweighted average linkage method. S, Similarity.

The aerobic strains were nonmotile, gram positive, and partially acid fast. Endospores were not formed, but after long incubation swollen cells (Fig. 2E and F) were observed.

The colonies were cream colored, sometimes with a tinge of pink. Red- or orange-pigmented colonies were never observed even after longer exposure to light. The colonies were irregularly wrinkled with lobate margins and were not easy to emulsify (Fig. 3D).

Physiology. The physiological properties of the cluster 1 strains are listed in Table 2, which includes all of the results used in the numerical assay. The features which are useful in distinguishing the strains in this cluster from the remaining strains tested are indicated. The following characters had a high discriminating value: no acid production from sucrose, trehalose, mannitol, or ethanol; no or poor growth with sodium benzoate, sodium citrate, proline, DL-norleucine, or testosterone as a sole carbon source but growth with L- α -alanine as a sole carbon and nitrogen source; no reduction of

nitrate to nitrite; no hydrolysis of urea, but degradation of esculin. The cluster 1 strains were further defined by their high susceptibilities to the antibiotic penicillin, rifampin, and vancomycin and the inhibitors crystal violet, sodium azide, phenol, phenyl ethanol, and potassium tellurite. On the other hand, they exhibited relatively low susceptibilities to lysozyme.

Chemotaxonomy. Purified cell walls of all cluster 1 strains contained *meso*-diaminopimelic acid and large amounts of arabinose and galactose, which is consistent with a type IV cell wall composition sensu Lechevalier and Lechevalier (18).

In thin-layer chromatography analyses of ethanol-diethyl ether extracts of the cluster 1 strains, mycolic acid spots were found. The R_f values of these spots were similar to those of the mycolic acid spots of *Nocardia asteroides* and two *Nocardia* sp. strains (serial no. 35 [*Nocardia flava*] and serial no. 36 [*N. polychromogenes*]). These mycolic

FIG. 2. *R. marinonascens* strain 3438W^T (= DSM 43752^T): slide microculture on yeast extract-malt extract-seawater medium at a growth temperature of 18°C. Interference contrast micrographs of substrate mycelium and microcolony development. (A) Developing mycelium after 4 days of cultivation. (B) Mycelium after 8 days. (C) Microcolony after 12 days of cultivation. (D) Colony margin after 16 days of cultivation. (E and F) Swelling of hyphal apices at the edges of some microcolonies after 24 days of cultivation. Bar = 10 μm.

acids could be classified as group C types according to the definition of Minnikin et al. (21). The mycolic acids of *R. rhodochrous* (serial no. 25) and *R. erythropolis* (serial no. 23) were determined as group B types.

The menaquinone pattern was also analyzed by thin-layer chromatography. Four cluster 1 strains (strains 2834W, 3438W, 4251W, and 4412W) were tested. These strains contained menaquinones with eight isoprene units and one hydrogenated double bond as the most prevalent type (Table 3).

The G+C contents of the DNAs were analyzed by thermal denaturation of five cream-colored strains (strains 1801W,

2834W, 2863W, 3438W, and 4251W); the values obtained ranged from 64.9 to 66.4 mol% (Table 3). Table 4 compares the diagnostic characteristics of cluster 1 with the properties of *Rhodococcus* and *Nocardia* species.

Distribution. Cream-colored nocardioform cluster 1 strains have so far been found only in marine habitats. They were isolated from the top layers of marine sediments from various sea sites including the Iberian Sea, the Bay of Biscay, the North Sea, and off northwest Africa, but they were more frequent in northern sea areas (Norwegian Sea, Barents Sea, North Atlantic Ridge, and off the Faeroe Islands). The highest numbers were obtained from the areas

FIG. 3. *R. marinonascens* strain 3438W^T (= DSM 43752^T) grown at 18°C on yeast extract-malt extract-seawater medium. (A through C) Slide microculture. Bar = 10 µm. (A) Beginning of fragmentation of the mycelium: mutually dislocating elements after 21 days of cultivation. Interference-contrast microscopy. (B) Progressive fragmentation at the edge of a colony after 22 days of cultivation. Phase-contrast microscopy. (C) Advanced stage of fragmentation into coccoid elements after 25 days of cultivation. Phase-contrast microscopy. (D) Elevated colony growth of a 21-day macroculture. Bar = 1 mm.

that were least influenced by land and from depths ranging up to 2,000 m (35).

DISCUSSION

Our numerical phenetic assay indicated that there is a close relationship between the red or orange marine isolates and the type strain of *R. rhodochrous*, as well as another strain of this species and *Rhodococcus* sp. strain DSM 43001. The latter two organisms were formerly labeled "*Nocardia rubra*" and "*Nocardia corallina*", respectively. The three reference strains cluster very closely together, confirming their reclassification as *Rhodococcus* species. The relatively high general similarity between four of the red or orange isolates and the marker strains is also supported by chemotaxonomic data; strain 4020R (serial no. 31) contains MK-8(H₂) as the prevalent menaquinone, mycolic acids of group B sensu Minnikin et al. (21), and DNA with a G+C content of 70.7 mol%. Supplementary tests will be necessary to establish whether the red or orange isolates are variants of *R. rhodochrous*.

Further work will also be needed to throw more light on the taxonomic status of red or orange strains 666R and 1732R.

In contrast to the red or orange marine nocardioform isolates, the cream-colored marine nocardioform isolates form a homogeneous, well-separated cluster which does not include any of the reference species. Regarding generic

status, the cluster 1 strains with G+C contents above 60 mol%, with nocardiomycolic acids of group C sensu Minnikin et al. (21), and with long-lasting well-developed mycelia can be clearly distinguished from the genera *Corynebacterium* and *Mycobacterium*. The numerical phenetic data, together with the data derived from chemical studies, indicate a close relationship between these strains and the genera *Nocardia* and *Rhodococcus*.

When the diagnostic characters of the genera *Rhodococcus* (5, 6, 12, 13) and *Nocardia* (12) are compared with the properties of cluster 1 strains (Table 4), the intermediate position of cluster 1 between *Rhodococcus* and *Nocardia* species becomes apparent. The affinity of this cluster to the genus *Nocardia* is underlined by morphological and colonial features, relatively low lysozyme susceptibility, and high susceptibility to phenol, sodium azide, crystal violet, and phenyl ethanol, as well as the presence of group C mycolic acids. Support for the classification of these strains in the genus *Rhodococcus* is based upon the lack of aerial mycelia and a diffusible exopigment, high susceptibility to penicillin, rifampin, and vancomycin, and the presence of menaquinones with eight isoprene units and one hydrogenated double bond.

The G+C content of the DNA is of secondary importance for generic classification into the taxa *Rhodococcus* and *Nocardia*. The G+C contents of the cluster 1 strains (64.9 to 66.4 mol%) fall within the narrow range for the genus

TABLE 3. Differentiating characteristics of *Rhodococcus* species

Characteristic	<i>R. marino-nascens</i> (17 strains)	<i>R. marinonascens</i> DSM 43752 [†]	<i>R. bronchialis</i>	<i>R. coprophilus</i>	<i>R. equi</i>	<i>R. erythropolis</i>	<i>R. glomerulus</i>	<i>R. luteus</i>	<i>R. maris</i>	<i>R. rhodni</i>	<i>R. rhodochrous</i>	<i>R. ruber</i>	<i>R. rubropertinctus</i>	<i>R. terrae</i>
Delayed fragmenting of primary mycelium ^a	100 ^b	+	-	+	-	-	-	-	-	-	-	-	-	-
Aerial hyphae	0	-	-	+	-	+	-	-	-	-	-	+	-	-
Colony margins entire	0	-	+	-	+	+	+	+	+	+	+	+	+	+
Irregularly wrinkled colonies ^a	100	+	-	+	-	+	-	-	-	-	-	-	-	-
Colonies pink, red, or orange	0	-	+	+	+	+	+	-	+	+	+	+	+	+
Colonies deep yellow	0	-	-	-	-	-	-	+	-	-	-	-	-	-
Colonies cream ^a	100	+	-	-	-	+	-	-	-	-	-	-	-	-
Growth at 10°C	100	+	-	+	+	+	+	+	+	+	+	+	+	+
Growth above 30°C ^a	0	-	+	+	+	+	+	+	+	+	+	+	+	+
Good growth with less than 0.5% salt ^a	0	-	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction ^a	23	-	+	+	+	+	+	+	+	+	+	+	+	+
Urease ^a	0	-	+	+	+	+	+	+	+	+	+	+	+	+
Degradation of tyrosine ^a	76	+	-	-	-	+	-	-	-	-	-	-	-	-
Degradation of adenine	0	-	-	-	+	+	-	-	-	-	-	-	-	-
Growth on sole carbon sources (0.1%, wt/vol)														
Sodium benzoate ^a	0	-	+	+	+	+	+	+	+	+	+	+	+	+
Sodium citrate	0	-	+	+	+	+	+	+	+	+	+	+	+	+
Sodium lactate	0	-	+	+	+	+	+	+	+	+	+	+	+	+
Sodium malate	82	+	+	+	+	+	+	+	+	+	+	+	+	+
Sodium succinate	88	+	+	+	+	+	+	+	+	+	+	+	+	+
Testosterone ^a	0	-	+	+	+	+	+	+	+	+	+	+	+	+
Growth on or acid ^g from sole carbon sources (1% wt/vol)														
Ethanol ^a	6	-	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	52	-	+	+	+	+	+	+	+	+	+	+	+	+
Inositol ^a	88	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose ^a	0	-	-	+	+	+	+	+	+	+	+	+	+	+
Mannitol ^a	0	-	+	+	+	+	+	+	+	+	+	+	+	+
Rhamnose	0	-	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	35	-	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose ^a	0	-	+	+	+	+	+	+	+	+	+	+	+	+
Trehalose ^a	0	-	+	+	+	+	+	+	+	+	+	+	+	+
Predominant menaquinone	8(H ₂)	8(H ₂)	9(H ₂)	8(H ₂)	8(H ₂)	8(H ₂)	8(H ₂)	8(H ₂)	8(H ₂)	8(H ₂)	8(H ₂)	8(H ₂)	8(H ₂)	9(H ₂)
Mycolic acid group ^{a,j}	C	C	D ^k	B ^f	Between groups A and B ^{d,i}	B ^k	C ^m	B ^g	B ^g	?	B ^k	B ^k	D or ? ^{f,k}	D ^k
G+C content of DNA (mol%) ^a	64.9-66.4	64.9	63-65 ⁿ	67.4-69.8 ^o	70.3-72.0 ^o	61-67 ⁿ	63-67 ^p	64.1 ^g	73.2 ^g	66 ^p	67.5-70.4 ^o	68.6-72.7 ^o	67.1-69.1 ^o	64-69 ⁿ

^a Distinguishing characteristics for *R. marinonascens*.^b Percentage of strains positive.^c +, Positive; -, negative; (-), predominantly negative; v, variable; ND, no data available.^d Data from reference 6.^e Data from reference 28.^f Data from reference 14.^g Data from reference 26.^h Data from reference 33.ⁱ Data from reference 11.^j Groups based on *R_f* values as described by Minnikin et al. (21).^k Data from reference 21.^l Data from reference 8.^m Data from reference 9.ⁿ Data from reference 25.^o Data from reference 23.^p Data from reference 24.

TABLE 4. Diagnostic characteristics of the genera *Rhodococcus* and *Nocardia* compared with the properties of cluster 1

Characteristic	<i>Rhodococcus</i> ^a	Cluster 1	<i>Nocardia</i> ^b
Aerobic	+	+	+
Gram positive	+	+	+
Primary mycelium	+	+	+
Well-branched mycelium	—	+	+
Soon fragmenting	+	—	—
Colony elevation irregular	—	+	+
Colonies easy to emulsify	+	—	—
Entire margin	+	—	—
Aerial hyphae	—	—	+
Diffusible exopigment	—	—	+
Wide range of carbon sources can be utilized	+	—	—
Lysozyme susceptible	+	(—)	—
Penicillin susceptible	+	+	—
Vancomycin susceptible	+	+	—
Rifampin susceptible	+	+	—
Cell wall type	IV	IV	IV
Mycolic acids ^c	Mostly group B	Group C	Group C
Prevalent menaquinone type	MK-8(H ₂) or MK-9(H ₂)	MK-8(H ₂)	MK-8(H ₄)
G+C content (mol%) as determined by chemical method	59–69		64–68
G+C content (mol%) as determined by thermal denaturation method	65–73	64–67	

^a Data from references 5, 6, 12, 13, and 23.^b Data from references 5, 10, 12, and 13.^c Analyzed and classified by the method of Minnikin et al. (21).

Nocardia (64 to 68 mol%, chemical method [10]), as well as the broad range for *Rhodococcus* species (59 to 69 mol%, chemical method [10], or 65 to 73 mol%, thermal denaturation method [23]).

Considering the above criteria for their value in generic classification, we believe that the menaquinone pattern is of greater classification value than the mycolic acids. Mycolic acid spots with R_f values similar to those designated as group C are found in strains other than *Nocardia* strains. Goodfellow et al. (9) showed that within *Rhodococcus*, subcluster 1b accommodates several strains containing group C mycolic acids. Two *Nocardia* sp. strains (serial no. 35 [*Nocardia flava*]) and serial no. 36 [*Nocardia polychromogenes*]) which were included as marker strains in this study are representative organisms with group C mycolic acids but no clear relationship to the genus *Nocardia*. Although the strains of cluster 1 resemble in this regard the two marker strains, the numerical phenetic data fail to show much affinity between strains of the two groups. In contrast to differences in mycolic acids, the menaquinone pattern is a more specific criterion for generic classification to *Nocardia* or *Rhodococcus*. The cluster 1 strains contain MK-8(H₂) as the prevalent quinone type. To our knowledge, MK-8(H₂) has never been found as a main menaquinone component in true *Nocardia* species, which usually possess MK-8(H₄). For this reason, and because of the lack of aerial mycelia and a diffusible pigment and susceptibility to penicillin G, rifampin, and vancomycin, we regard the genus *Rhodococcus* as an acceptable taxonomic niche for cluster 1. When characteristics of the previously recognized species of this genus are compared with the morphological, physiological, and chemotaxonomic properties of strains in cluster 1 (Table 3), it is obvious that the cluster 1 strains cannot be equated with

any of the previously established *Rhodococcus* species. Therefore, we consider the strains of cluster 1 a new species within the genus *Rhodococcus*. Since these strains occur exclusively in marine habitats and possess general features of bacteria indigenous to the sea (36; E. Helmke, Dr. thesis, Universität Braunschweig, Federal Republic of Germany, 1980), we name this organism *Rhodococcus marinonascens* sp. nov. (ma. ri. no. nas'cens. L. adj. *marinus* of the sea; L. part. adj. *nascens* nascent, born; M. L. part. adj. *marino-nascens* nascent in the sea). The type strain of this species is 3438W (= DSM 43752). A taxonomic description is given below.

Description of *Rhodococcus marinonascens* sp. nov. Aerobic, slow-growing nocardioform actinomycetes that are isolated from marine bottom sediments and are unable to develop in freshwater media and at temperatures above 28°C. Optimal growth occurs around 20°C and in media with a seawater content of 75 to 100% or an equivalent salt concentration. *R. marinonascens* is gram positive, nonmotile, and partially acid fast and develops well-branched mycelia which fragment into bacillary and coccoid elements on solid media after 14 or more days of incubation. The colonies are cream-colored with a tinge of pink, irregularly wrinkled with lobate margins, and not easy to emulsify. Aerial mycelia and extracellular pigments are not formed. *R. marinonascens* has a type IV cell wall composition and contains group C mycolic acids sensu Minnekin et al. (21) and MK-8(H₂) as the main menaquinone component. The DNA base composition is 64.9 mol% G+C.

R. marinonascens produces acid from glucose, fructose, mannose, inulin, and inositol. Unlike many other *Rhodococcus* species, under the specific test conditions which we used, the strains exhibit no acid formation from sucrose, trehalose, mannitol, or ethanol and only weak acid production from dextrin. Also in contrast to many other *Rhodococcus* species, *R. marinonascens* cannot utilize sodium benzoate, sodium citrate, L-proline, or testosterone as a sole carbon source, but L- α -alanine serves as a sole carbon and nitrogen source. Catalase is formed, whereas oxidase, urease, and nitrate reductase are not produced; esculin can be hydrolyzed. *R. marinonascens* is further characterized by high susceptibility to the antibiotics penicillin G, rifampin, and vancomycin and the inhibitors crystal violet, sodium azide, potassium tellurite, phenyl ethanol, and phenol; on the other hand, this organism is relatively resistant to lysozyme.

Type strain: 3438W (= DSM 43752).

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