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REVIEW

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Taxonomy and biotransformation activities of some deep-sea actinomycetes

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Abstract Deep-sea soft sediments from trench systems and depths in the northwestern Pacific Ocean ranging from less than 300 to 10 897 m in depth have been analyzed for three target genera of actinomycetes: *Micromonospora*, *Rhodococcus*, and *Streptomyces*. Only culturable strains, recovered at atmospheric pressure on selective isolation media, have been examined to date. Maximum recoveries of culturable bacteria were greater that 10^7/ml wet g sediment, but actinomycetes comprised a small proportion of this population (usually less than 1%). The target actinomycetes were isolated at all depths except from the Mariana Trench sediments. Actinomycete colonies were defined initially on the basis of colony morphologies, and preliminary identification then was made by chemotaxonomic tests. Pyrolysis mass spectrometry (PyMS) of deep-sea mycolic acid-containing actinomycetes gave excellent correspondence with numerical (phenetic) taxonomic analyses and subsequently was adopted as a rapid procedure for assessing taxonomic diversity. PyMS analysis enabled several clusters of deep-sea rhodococci to be distinguished that are quite distinct from all type strains. 16S rRNA gene sequence analysis has revealed that several of these marine rhodococci have sequences that are very similar to certain terrestrial species of *Rhodococcus* and to *Dietzia*. There is evidence for the intrusion of terrestrial runoff into these deep trench systems, and the inconsistency of the phenotypic and molecular taxonomies may reflect recent speciation events in actinomycetes under the high-pressure conditions of the deep sea. The results of DNA-DNA pairing experiments point to the novelty of *Rhodococcus* strains recovered from hadal depths in the Izu Bonin Trench. Biotransformation studies of deep-sea bacteria have focused on nitrile compounds. Nitrile-metabolizing bacteria, closely related to rhodococci, have been isolated that grow well at low temperature, high salt concentrations, and high pressures, suggesting that they are of marine origin or have adapted to the deep-sea environment.

Key words Deep sea · Actinomycetes · Selective isolation · Pyrolysis mass spectrometry · Nitrile transformations · High pressure

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Introduction

Microbial diversity as a source of innovation for biotechnology is a major interest of our laboratory (Bull et al. 1992), and it is in this context that the deep-sea environment has proved such an attractive one to explore. Despite the fact that 60% of the Earth's surface is covered by seas of depths exceeding 2000 m, the inventory of microorganisms in the deep sea is very incomplete. However, recent surveys of the open oceans, hydrothermal vents, and the deepest ocean floor (DeLong 1997; Jollivet 1996; Takami et al. 1997) have revealed unsuspected diversity and novelty in their microbiota. Our research has focused on bacteria of soft deep-sea sediments.

In this paper we report the results of taxonomic and biotransformation studies aimed at detecting novelty among deep-sea bacteria. Actinomycetes were chosen for this study because (i) they have been reported previously in marine ecosystems, (ii) the taxonomy of terrestrial actinomycetes is well established and provides an excellent background against which to assess novelty in the deep sea, and (iii) they have singular value from a biotechnological perspective. Although reports of actinomycetes in marine sediments go back several decades, for the most part the reference to actinomycetes was incidental, or the sediments were from shallow inshore locations (Goodfellow and Haynes 1984; Jensen et al. 1991; Takizawa et al. 1993).

Moreover, the few deep-sea sediments examined contained relatively low numbers of actinomycetes (Weyland 1969; Helmke and Weyland 1984; Goodfellow and Haynes 1984), and it has not been established whether these were truly indigenous organisms or simply residual populations from terrestrial wash-in. Consequently, we have analyzed sediments obtained from the complete range of bathyal, abyssal, and hadal depths and have used taxon-designed selective procedures for isolating streptomycetes, micromonosporae, and mycolate (mycolic acid-containing) actinomycetes. We have assessed the terrestrial wash-in of actinomycetes by detecting the presence of thermoactinomycetes in sediment samples (Attwell and Colwell 1984), while profiling isolates in terms of their temperature, salinity, and pressure requirements and tolerances has provided evidence for an indigenous actinomycete community in deep seas.

This phase of our deep-sea actinomycete program has focused on culturable, aerobic, barotolerant populations. To circumscribe large numbers of isolates rapidly, we have deployed pyrolysis mass spectrometry as a means of detecting phenotypic novelty, and evaluated its reliability by making polyphasic taxonomic analyses of the clusters that were generated. The potential of deep-sea actinomycetes as sources of novel biotransformations was explored with respect to their nitrile-metabolizing activities. Such activities are exploitable for chemical syntheses and for biotreatment of toxic environmental chemicals and are well documented in terrestrial bacteria (Wyatt and Linton 1988).

Culturable actinomycetes

Culturable bacteria, isolated on 10% tryptic soy agar formulated with artificial seawater (Takizawa et al. 1993), were recovered from all the deep-sea sites examined (Table 1). There was no obvious correlation between population density and depth. The highest counts were obtained from both shallow sites (Sea of Japan, Sagami and Suruga Bays) and certain abyssal and hadal sites in the Ryukyu and Japan Trenches, but even at the extreme depth of the Challenger Deep nearly 10^3 bacteria per milliliter of wet sediment were recovered. Numbers of the target actinomycetes (Table 1) in most cases were approximately 10^3/ml wet sediment, i.e., less than 1% of the total culturable bacteria; in one case, however (Japan Trench, 6455 m), actinomycetes constituted nearly 9% of this total. These numbers are comparable with

Table 1. Total culturable bacteria and abundance of actinomycetes in deep-sea sediments

Sampling location	Depth (m)	Total cfu/ml wet sediment	Actinomycetes/ml wet sediment		
			Mycolate	Other	Total
Japan Sea	289	2.5×10^6	2.0×10^3	2.3×10^3	4.3×10^3
Sagami Bay	1168	1.9×10^6	2.0×10^2	3.2×10^2	5.2×10^2
Suruga Bay	1151	1.3×10^6	6.7×10^2	4.0×10^3	4.7×10^3
	1487	1.2×10^7	0	1.0×10^3	1.0×10^3
	1948	ND	2.4×10^3	3.3×10^3	5.7×10^3
Okinawa Trough	1393	2.0×10^3	3.0×10^2	1.0×10^3	1.3×10^3
Archipelago, SE Okinawa	2859	1.0×10^3	0	0	0
Ryukyu Trench	5110	2.7×10^7	0	0	0
	5425	3.5×10^6	0	0	0
	6600	5.0×10^2	0	0	0
Izu-Bonin Trench	2679	3.0×10^6	5.0×10^3	3.3×10^2	5.3×10^3
	4056	ND	1.0×10^3	0	1.0×10^3
	4644	2.0×10^5	0	0	0
	4739	1.2×10^6	0	0	0
	6390	2.0×10^5	1.0×10^3	0	1.0×10^3
	6499	1.1×10^6	2.0×10^3	3.3×10^2	2.3×10^3
Japan Trench	4418	2.1×10^5	1.3×10^3	3.3×10^2	1.6×10^3
	6048	2.0×10^5	3.3×10^2	0	3.3×10^2
	6142	1.1×10^7	0	0	0
	6300	1.3×10^6	0	0	0
	6372	8.3×10^4	0	0	0
	6455	1.5×10^4	2.0×10^3	0	2.0×10^3
	6475	7.4×10^6	0	0	0
Mariana Trench	10898	8.0×10^2	0	0	0

ND, not determined.
Enumeration of culturable bacteria was made on 10% trypic soy agar formulated with artificial seawater (Takizawa et al. 1993); incubations were made at atmospheric pressure and 30°C. Isolation of target actinomycetes was made using the following selective media: (i) *Rhodococcus*: Münz medium containing 1% (v/v) paraffin (Nesterenko et al. 1978), M3 medium (Rowbotham and Cross 1977); (ii) *Micromonospora*: starch-casein-nitrate containing novobiocin, and cellulose-asparagine media (Goodfellow and Haynes 1984); (iii) *Streptomyces*: starch-casein-nitrate medium (Küster and Williams 1964). All selective media were supplemented with cycloheximide (100 mg/ml) to suppress fungal growth.

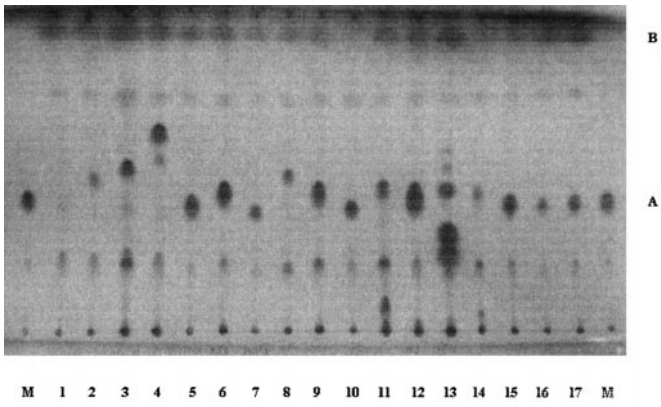

Fig. 1A,B. Representative mycolic acid thin-layer chromatography (TLC) profiles of type strains and deep-sea mycolate actinomycetes. **A** Mycolic acid; **B** nonhydroxylated fatty acid methyl esters. *1*, Negative control (nonmycolate actinomycete); *2*, *Nocardia asteroides*; *3*, *Gordona bronchialis*; *4*, *Tsukamurella paurometabola*; *5*, *Dietzia maris*; *6*, *Rhodococcus rhodochrou*; *7*, *R. marinonascens*; *8*, IBT2679/051; *9*, IBT2679/068; *10*, OKT1393/078; *11*, SGB1168/117; *12*, SRB1948/A07; *13*, SRB1948/E04; *14*, SJS0289/JS3; *15*, IBT6499/047; *16*, JTS6455/242b; *17*, JTS4418/260b. Cell-wall mycolic acids were extracted and analyzed by TLC (Minnikin 1988). Detection was made with molybdophosphoric acid (5% w/v) followed by development of the TLC plates at 180°C for 10 min

those recorded in relatively shallow marine sediments (Chesapeake Bay, Takizawa et al. 1993; coastal sediments in the UK, Goodfellow and Haynes 1984), and the few deep-sea sediments that have been investigated (Weyland 1969; Goodfellow and Haynes 1984). In the sediments we have examined, the dominant actinomycetes appear to belong to mycolate taxa.

The highest counts of *Thermoactinomyces* strains also were found in shallow-sea sediments (Colquhoun et al. [in press]), with maximum numbers recovered at 289 m from the Sea of Japan (1.7×10^5 cfu/ml wet sediment). Such data are consistent with the ingress of terrigenous microorganisms into marine ecosystems, and the population sizes are similar to those reported for estuarine waters and sediments (Attwell and Colwell 1984). At deep-sea sites, the numbers of *Thermoactinomyces* were several orders of magnitude lower, ranging from 10% to less than 1% of the total culturable actinomycetes, or were completely absent (Okinawa Trough, Ryukyu and Mariana Trenches; Colquhoun et al. [in press]). Thus, the deep-sea sites included in this study varied in the extent to which they were sinks for terrestrial material.

Preliminary grouping of isolates was made on the basis of colony characteristics, microscopy, and, in the case of putative rhodococci, mycolic acid profiles. *Rhodococcus* type strains and deep-sea isolates produced a single mycolic acid spot on thin-layer chromatography (TLC) plates (R_f 0.39–0.47) and were distinguishable from strains of *Gordona* (R_f 0.52) and *Tsukamurella* (R_f 0.63), and mycobacteria (e.g., Suruga Bay isolate SRB1948/E04) that produced multiple mycolic acids (Fig. 1).

Taxonomy of deep-sea mycolata

Curie-point pyrolysis mass spectrometry (PyMS) was used as the preferred means for sorting the putative rhodococci into clusters. The advantages of PyMS in this context are its speed, sensitivity, and capacity to handle large numbers of strains. Before deciding on the use of PyMS, it was validated against other methods for sorting the deep-sea rhodococci by comparison with an extensive numerical taxonomic (NT) analysis. PyMS and NT analyses, made independently in different laboratories, produced complete congruence of clusters (Colquhoun et al., unpublished data). Our subsequent strategy for circumscribing putative rhodococcal isolates was to use the following polyphasic taxonomic approach: (i) PyMS to define the range of phenotypic diversity, (ii) 16S rDNA sequencing to establish phylogenetic relationships, and (iii) DNA-DNA pairing to resolve questions of species identity. The following selection of data illustrate the scope and efficacy of the approach.

The dendrogram shown in Fig. 2 was produced from the PyMS analysis of 39 deep-sea isolates recovered from a range of sites and depths, and 16 type strains of mycolate actinomycetes. Three major (A, C, D) and two minor (B, E) clusters were defined by this initial analysis. Intracluster variation is quite small, suggesting the close relationship between strains within such clusters. The percentage similarity values are derived from Mahanalobis distances for each pair of strains, and although these values define only a portion of interstrain discrimination (Magee 1994), when the data set contains reference strains it is possible to establish the relative closeness of unknown to known organisms. It is important to point out, however, that even small intracluster discriminations of the sort seen in Fig. 2 may be highly relevant for biotechnology screening purposes (Bull et al. 1992).

The major clusters shown in Fig. 2 were progressively reanalyzed by removing outlying strains from the data set. Thus, cluster C, which contained isolates from various depths in the Izu Bonin and Japan Trenches, was differentiated into four subclusters. Subcluster C2 isolates were grouped with *Dietzia maris* while the Japan Trench isolates (JTS#) were clearly differentiated on the basis of depth (C4, 4418 m; C1, 6048 m; C3, 6455 m) (Colquhoun et al. [in press]).

Fifteen deep-sea isolates representative of the PyMS clusters, together with a number of PyMS outliers, were subjected to BioLog™ (BioLog, Haywood, CA, USA) analysis. Also included in the analysis were (i) Izu Bonin Trench isolates (IBT2679/024 and IBT2679/068) that had been stored at −70°C in glycerol for 2 years, and (ii) a number of recently isolated terrigenous strains of *Rhodococcus*. The results of this analysis are shown in Fig. 3. While clusters generated by BioLog analysis were much more heterogeneous compared to those by PyMS, clusters A, C, and D were recognizable. The poorer resolving power of BioLog clearly reflects the smaller number and narrower range of phenotypic characters on which it is based. The storage of isolates at −70°C appeared not to affect their

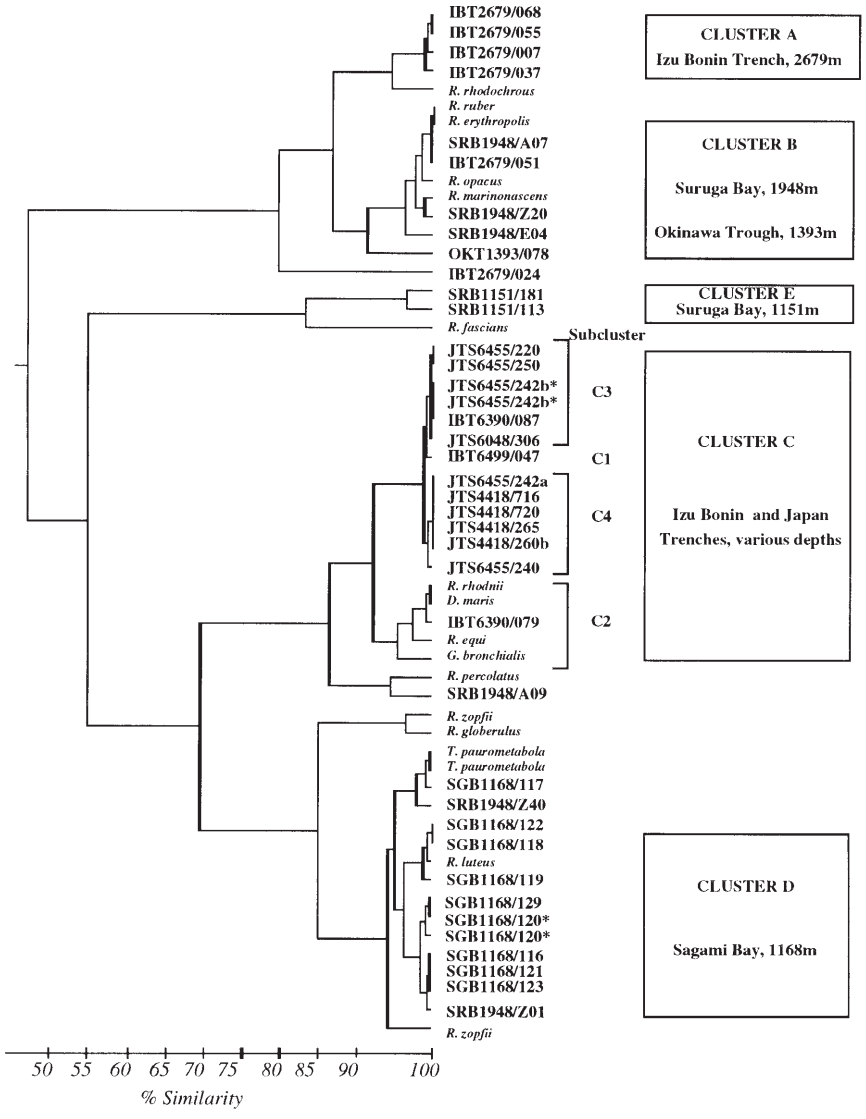

Fig. 2. Dendrogram derived from pyrolysis mass spectrometry (PyMS) data for deep-sea and type strain actinomycetes isolated from diverse sampling sites. Deep-sea isolates, type strains of *Rhodococcus*, and other mycolate actinomycete genera were grown on GYE agar (Gordon and Mihm 1962) for 5 days at 30°C. Samples for pyrolysis were prepared and analyzed with a Horizon RAPyD 400 pyrolysis mass spectrometer (Horizon, Heathfield, UK) using methods developed for actinomycete classification (Sanglier et al. 1992). Triplicate samples were analyzed for each isolate and type strain. Ranked masses were subjected to principal component and canonical variate analyses using the GENSTAT statistical package (Numerical Algorithms Group, Oxford, UK). Similarities were calculated as Mahalanobis distances for each pair of strains, and clusters were defined using the unweighted pair group method with arithmetic averages (UPGMA; Sneath and Sokal 1973). * Duplicat samples showing the reproducibility of the analyses

phenotypic properties, as evidenced by the very close grouping of glycerol-stored and agar-passaged replicates of the same strain (cf. IBT2679/024g and 024, and IBT 2679/068g and 068, respectively; see Fig. 3). BioLog analysis of recently isolated terrigenous rhodococci was made to check that the PyMS clustering of deep-sea strains away from the type strains did not simply reflect type strain degeneration during prolonged laboratory cultivation. The terrigenous strains were mainly collected into a large heterogeneous group at the 80% similarity level that contained members of PyMS clusters A, B, and C; they clustered closely with terrigenous type strains or remained separated from their nearest neighbors, suggesting that the discrimination of the deep-sea isolates was not artifactual. BioLog analysis recovered the nitrile-transforming rhodococci as a minor cluster whose identity was supported by 16S rDNA sequencing (see following).

16S rDNA gene sequencing was done on representatives of the major PyMS clusters together with a number of deep-sea isolates of indeterminate PyMS grouping (SRB1151/113, SRB1948/A07, SRB1948/E04, OKT1393/078, IBT2679/051, and SJS0289/JS1) according to the procedure reported previously (Kato et al. 1997). The phylogenetic relationships between the deep-sea isolates and 65 type strains of 7 genera of mycolata are shown in Fig. 4. *Corynebacterium*, *Gordona*, *Mycobacterium*, and *Tsukamurella* formed phylogenetically distinct clades whereas it is clear that the status of the *Rhodococcus-Nocardia* taxonomic group requires further resolution. Our analysis confirms the view that *Nocardia* constitutes a clade within the *Rhododoccus* radiation (Rainey et al. 1995).

The 16S rDNA analysis provides strong evidence for the occurrence of novel mycolate taxa in the deep seas. The Okinawa Trough isolate, strain OKT1393/078, appears to be a new species of *Corynebacterium*, while isolates from the Izu Bonin Trench (IBT2679/051, cluster B) and Suruga Bay (SRB1151/113, cluster E, and SRB1948/E04) are probably new *Gordona* and *Mycobacterium* species, respec-

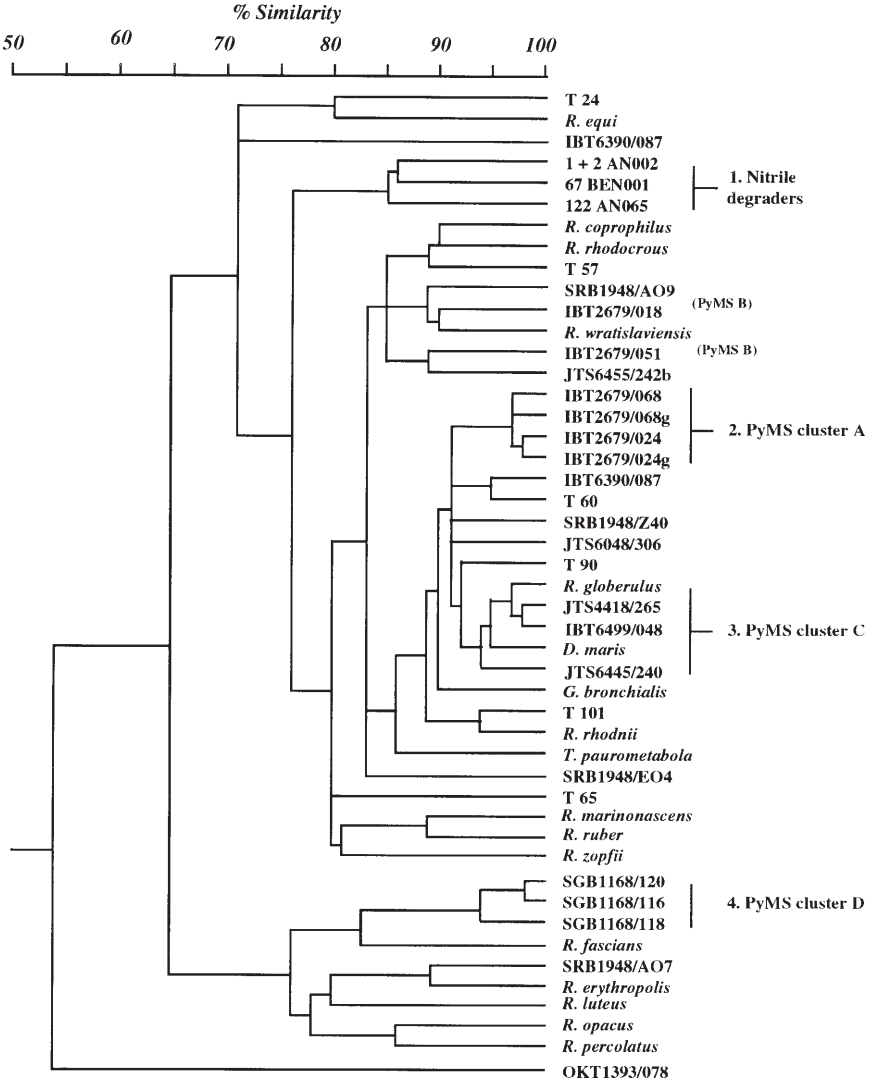

Fig. 3. Dendrogram derived from UPGMA cluster analysis of BioLog data for representative deep-sea and terrestrial actinomycetes and type strains. *T-series*, rhodococci recently isolated from terrestrial sites. Substrate utilization was tested against the selected strains ability to oxidize a panel of 95 carbon sources (BioLog, Haywood, CA, USA). Tests were run in duplicate and the data subject to cluster analysis using the UPGMA algorithm (MicroLog™3 software)

tively. The status of isolate SJS0289/JS1 from the Sea of Japan is especially interesting, and its phylogenetic depth is suggestive of a new genus separated from the *Gordona* clade. The 16S rDNA data confirmed that among PyMS cluster C (including JTS6048/306 and JTS6455/250) are strains of the authenticated deep-sea actinomycete *Dietzia maris*. Finally, the 16S rDNA data point to PyMS cluster A and D isolates as terrigenous actinomycetes that have entered the marine environment by wash-in. DNA-DNA pairing experiments according to the procedure of Ezaki et al. (1989) confirmed cluster D isolates as *Rhodococcus luteus*. The PyMS cluster A isolates (IBT2679/024 and IBT2679/068), which 16S rDNA data place with *R. rhodochrous* and *R. coprophilus*, were identical to each other on the basis of DNA pairing analyses, and the very low similarity (20%) proved that they were not *R. rhodochrous*. They showed 40% similarity to *Rhodococcus coprophilus*, a value well below the 70% similarity accepted as defining a genomic species (Wayne et al. 1987). The 40% value is equivocal and could indicate a distinct genomic species, or, a DNA group within the *R. coprophilus* taxon; however, it does caution against a too rigid reliance on 16S rDNA evidence for circumscribing species. This point has been stressed by Fox et al. (1992), who comment that although 16S sequences are valuable for distinguishing genera and well-resolved species, very recently diverged species, of the sort we suspect among deep-sea rhodococci, may not be recognizable.

Nitrile-transforming activities of deep-sea rhodococci

We have isolated a number of bacteria from marine sediments by direct and batch enrichment culture on acetonitrile or benzonitrile. All these strains contained mycolic acids with similar mobilities on TLC plates to the reference strains of *Rhodococcus*, suggesting that they were likely to be members of this genus. Strains 122-AN065 and 67-BEN001, recovered from depths greater than 5000 m (Ryukyu Trench, 5425 m and Japan Trench, 6475 m, respec-

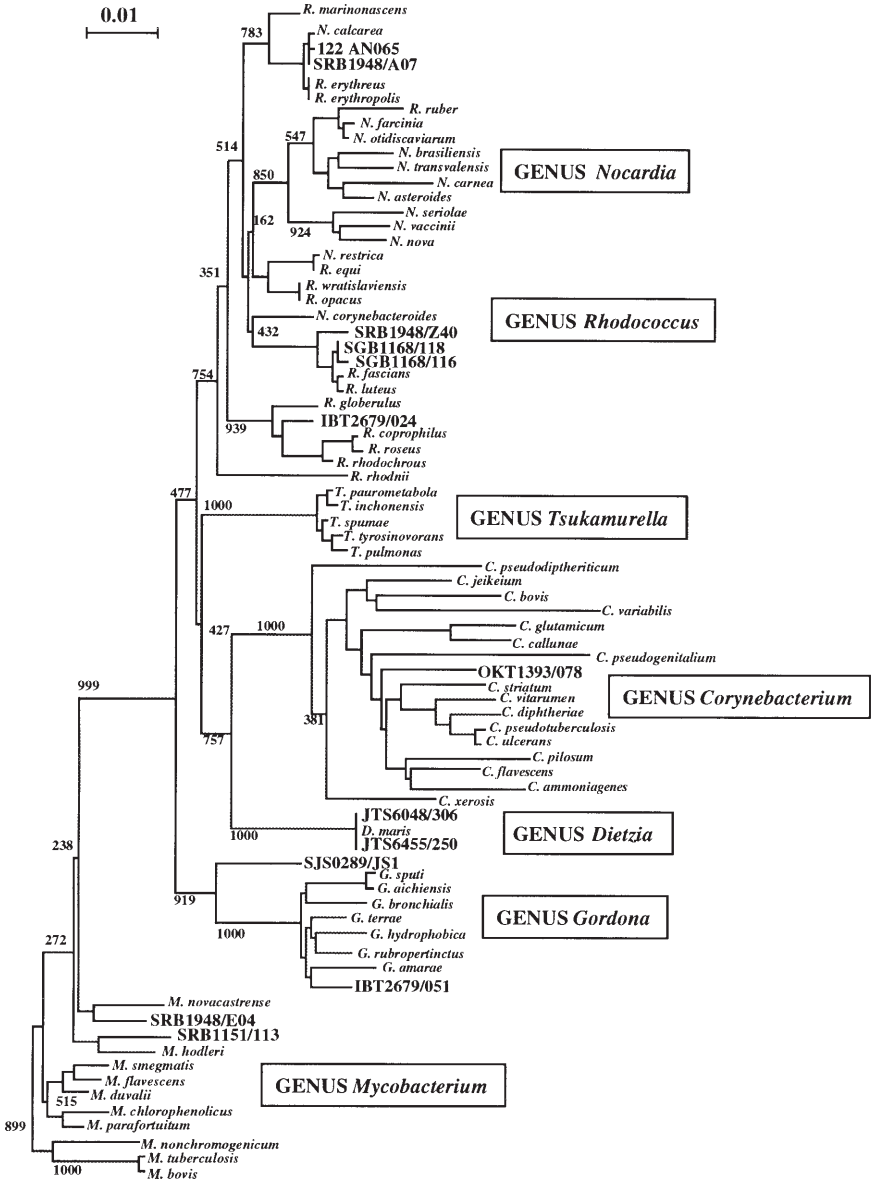

Fig. 4. Phylogram based on the comparison of 16S rDNA sequences of mycolate actinomycete type strains and selected deep-sea isolates. Bootstrap values are given for the likelihood of evolution from each major node. *Bar* indicates inferred substitution per 100 nucleotides. The 16S rDNA sequences of the isolated strains have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases. Strain names and accession numbers are as follows: IBT2679/024, AB010902; IBT2679/051, AB010903; JTS6048/306, AB010904; JST6455/250, AB010905; OKT1393/078, AB010906; SGB1168/116, AB010907; SGB1168/118, AB010908; SJS0289/JS1, AB010909; SRB1151/113, AB010910; SRB1948/A07, AB010911; SRB1948/E04, AB010912; SRB1948/Z40, AB010913

tively), grew consistently well on the nitriles on which they were isolated. 16S rDNA sequencing of strain 122-AN065 showed it to have 99.8% sequence homology with *Nocardia calcarea* and 99.7% homology with *Nocardioides simplex* (Rainey et al. 1995), which was reclassified as *R. erythropolis*. Strain 67-BEN001 had an identical 16S rDNA restriction length fragment polymorphism pattern to 122-AN065 and also is likely to have a similar sequence identity.

Strain 122-AN065 grew on both aliphatic (acetonitrile) and aromatic (benzonitrile) nitriles, although growth was slower on the latter compound. The specific growth rate on 20 mM acetonitrile at 30°C was 0.21 h^{-1} compared to 0.02 h^{-1} on acetonitrile at 4°C and 0.08 h^{-1} on 10 mM benzonitrile at 30°C. Growth was fastest at acetonitrile concentrations of 50 and 100 mM, was severely inhibited at concentrations in excess of 500 mM, and ceased completely at 2 M (Heald et al., manuscript in preparation).

Strain 67-BEN001 also was able to grow on aromatic and aliphatic nitriles as sole carbon sources. Growth occurred at concentrations between 1 and 30 mM benzonitrile but not at 50 mM, the maximum solubility of the substrate in minimum medium. The maximum specific growth rate on benzonitrile minimum medium supplemented with yeast extract (5 $mg\,l^{-1}$) was 0.12 h^{-1}. Acetonitrile-grown, resting cells of strain 67-BEN001 metabolized acetonitrile and benzonitrile with the production of the corresponding acid and ammonia. During these transformations, amides were not detected in the reaction mixtures although the strain was able to metabolize acetamide and benzamide, thus indicating amidase activity. Biotransformation data suggested that strain 122-AN065 had a nitrile hydratase-amidase system in which amidase activity was higher than nitrile hydratase activity when the strain was grown on a nitrile substrate; however, when growth was on succinate, the amidase activity was lower than the hydratase, and thus amide

Table 2. Hydrolysis rates of nitrile and amide substrates by deep-sea *Rhodococcus* strains 67-BEN001 and 122–AN065

Substrate	Nitrogen release by strain 67-BEN001 after growth on					Strain 122-AN065	
	Growth	Benzonitrile	Glycerol	Benzonitrile/yeast	Acetonitrile	Growth	Nitrogen release after growth on acetonitrile
Acetonitrile (C2)	Y	184	124	112	403	Y	100
Acrylonitrile	–	–	–	–	–	N	64
4-Aminobenzonitrile	–	–	–	–	–	N	18
Benzonitrile	Y	100	100	100	100	Y	50
Butyronitrile (C4)	Y	940	364	630	–	Y	840
Chloroacetonitrile	N	438	270	–	–	N	76
Crotononoitrile	N	64	17	–	–	Y	36
3-Cyanopyridine	N	126	–	–	–	N	0
Fumaronitrile	–	–	–	–	–	N	0
Isovaleronitrile	–	143	71	212	–	Y	–
Malononitrile	–	–	–	–	–	N	0
Methoxyacetonitrile	N	–	189	–	–	N	38
D,L-Mandelonitrile	–	–	–	–	–	N	0
2-Nitrobenzonitrile	–	–	–	–	–	N	20
Propiononitrile (C3)	Y	942	257	435	–	Y	70
Succinonitrile (C6)	Y	–	–	–	–	Y	379
Valeronitrile (C5)	Y	1636	362	–	–	Y	815
Acetamide	Y	420	124	630	–	Y	>4000
Acrylamide	–	–	103	–	–	–	–
Benzamide	Y	–	68	–	–	–	–
Lactamide	Y	–	322	–	–	–	1124
Salicylamide	–	–	0	–	–	–	37

Rates of hydrolysis were normalized to the rate of hydrolysis of benzonitrile (nmol min^{-1} mg^{-1} protein) by strain 67-BEN001 (grown on benzonitrile, 162; glycerol, 292; benzonitrile/yeast, 200; acetonitrile, 302) and acetonitrile by strain 122-AN065 (grown on acetonitrile, 380). –, not determined; Y, growth on compound; N, no growth on compound.
Values in parentheses indicate carbon number of straight-chain aliphatic compounds.

accumulated transiently. Strain 67-BEN001 when grown on benzonitrile, acetonitrile, glycerol, or benzonitrile plus yeast extract ($5 mg l^{-1}$) released ammonia from both acetonitrile and benzonitrile. Acetonitrile was transformed at a faster rate than benzonitrile. The transformation of acetamide by these bacteria again was indicative of the presence of amidase activity. Resting cell suspensions also were able to metabolize benzamide. When strain 67-BEN001 was grown on benzonitrile without yeast extract, amide did not accumulate, and the rate of benzonitrile transformation was approximately tenfold slower than that in the presence of yeast extract.

Cell-free extracts of strains 122-AN065 and 67-BEN001 grown on acetonitrile and benzonitrile, respectively, were electrophoresed on native PAGE (polyacrylamide) gels and stained with anthranilonitrile. A single distinct fluorescent band, visible under UV light, was produced by both strains as a consequence of conversion of the nitrile to fluorescent amide or acid products. This result was indicative of a single nitrile-metabolizing enzyme system present in each strain, although it did not rule out the possibility of the coexistence of a nitrile hydratase-amidase and nitrilase that comigrated or that the activity of one of these enzymes was lost during extraction.

The nitrile-metabolizing enzyme of strain 67-BEN001 has been separated from the amidase activity by ion-exchange chromatography (Heald et al., manuscript in preparation). The nitrile active fraction when electrophoresed by SDS-PAGE was shown to constitute two 25-kDa bands; this is the same size as the a- and b-subunits of nitrile hydratase (Yamada and Kobayashi 1996). HPLC analysis of benzonitrile transformation mixtures produced by the nitrile active fraction revealed benzamide as the predominant product, confirming the presence of a nitrile hydratase-amidase system in this bacterium.

The nitrile-transforming activities of both deep-sea strains had broad susbstrate specificities (Table 2), with a preference for aliphatic nitriles. Valeronitrile (C5) and butyronitrile (C4) were the preferred substrates and were metabolized much faster than either acetonitrile or benzonitrile. Strain 67-BEN001 also had a high rate of hydration of propiononitrile (C3). Dinitriles (succininitrile), chloroacetonitrile, and a number of amides were also metabolized. The two deep-sea rhodococci characterized in this study differ from the only other reported marine nitrile-transforming *Rhodococcus* (*R. erythropolis* strain BL1; Langdahl et al. 1996) in that the latter was isolated from coastal sediments and was unable to metabolize benzonitrile.

Effects of simulated deep-sea conditions on growth of deep-sea rhodococci

The effect of NaCl concentration, temperature, and pressure (over ranges similar to those of the deep-sea environment) on growth were investigated. The marine sediments

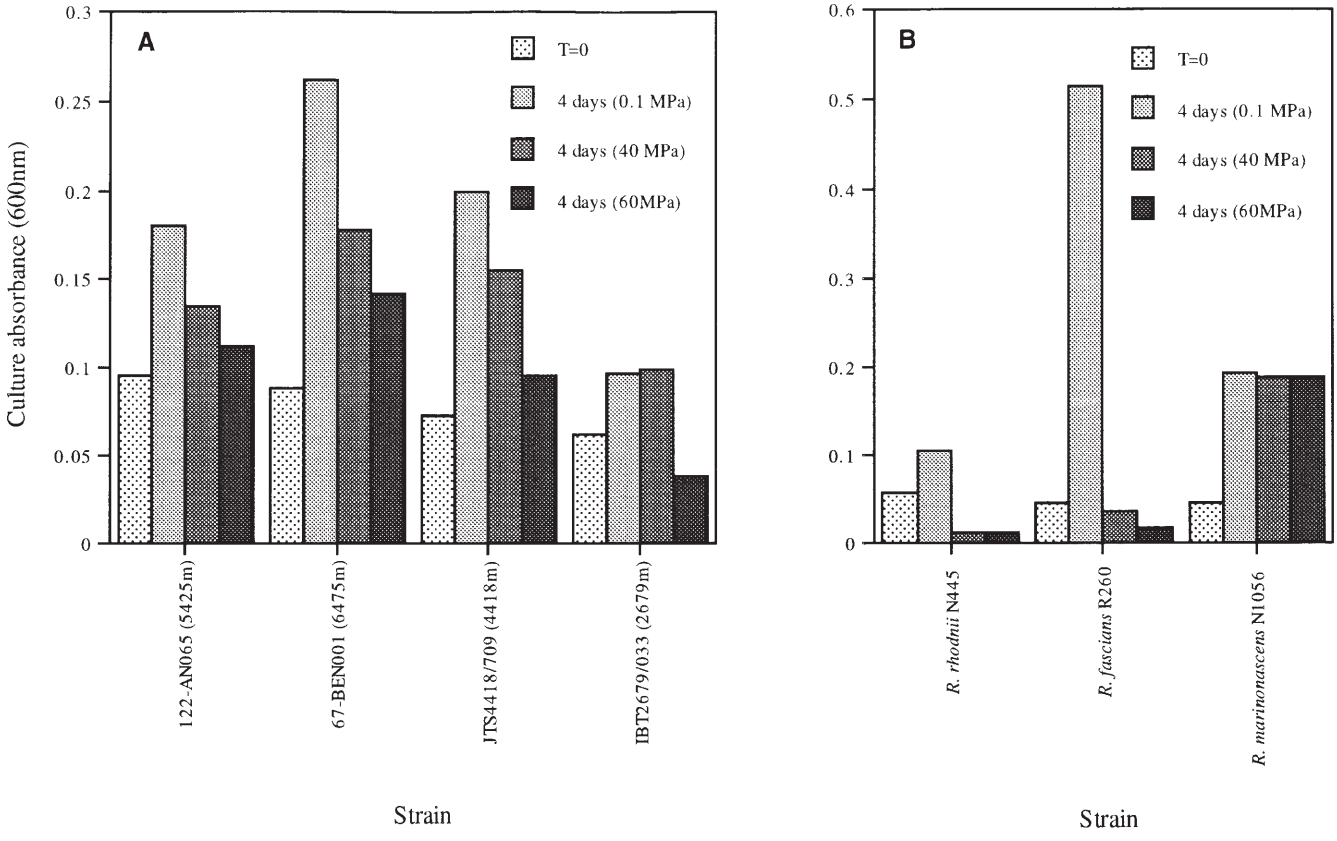

Fig. 5A,B. Growth of deep-sea rhodococci (**A**) and selected type strains of *Rhodococcus* (**B**) at atmospheric pressure (1 atm), and at high pressures, 40 and 60 MPa. Control cultures were incubated at atmospheric pressure. All experiments were performed for 4 days at 30°C. $T = 0$, culture absorbance of the inoculum. Growth at hyperbaric pressures was determined in 1.5-ml sterile autosampler vials containing 0.5 ml of the oxygen carrier Fluorinet FC-72 (Sigma) without a gaseous headspace. Before to use Fluorinet was degassed under vacuum, oxygenated for 2 h, and filter-sterilized. Vials were incubated in hydraulically pressurized vessels constructed from Inconel 625 high-strength nickel-chromium alloy (Stott and Herbert 1986)

from which these strains were isolated had total halide concentrations ranging from 2.5% to 3.2%. Nitrile-transforming strains grew on 20 mM acetonitrile in liquid minimum medium at salinities from 0% to 4% (w/v).

The deep-sea abyssal depths are at a constant temperature of 2°–4°C (Yayanos 1995). All nitrile-transforming bacteria were isolated at 30°C. Strain 122-AN065 grew on acetonitrile at 4°C, although the growth rate was only 10% of that at 30°C. Acetonitrile transformations rates also were approximately 10 fold lower at 4°C. Strain 67-BEN001 also grew on benzonitrile at 4°C, but transformation rates were more than 30 fold lower than at 30°C.

The effect of elevated hydrostatic pressure on growth in glucose-yeast extract medium of deep-sea strains from a range of depths is shown in Fig. 5. Strains 122-AN065 and 67-BEN001 isolated from 5425 and 6475 m, respectively, grew at a pressure of 40 MPa. At 60 MPa growth still occurred, but the isolate from the shallower depth was more sensitive to this higher pressure. The terrestrial strains *R. rhodnii* and *R. fascians* did not grow at 60 MPa or at 40 MPa and, after incubation at elevated pressures, cell lysis occurred. Interestingly, a culture collection type strain, *R. marinonascens* (Helmke and Weyland 1984), originally isolated from marine sediments as deep as 2000 m but which had been maintained at atmospheric pressure for many years, also was resistant to high pressure and grew at 40 and at 60 MPa. The specific responses of the deep-sea rhodococci to conditions found in the marine environment strongly suggest that they are marine in origin. This conclusion is supported by the low numbers or complete absence of *Thermoactinomyces* in the deep ocean trenches.

The diversity of actinomycetes in deep-sea sediments and their ability to synthesize biotechnologically important enzymes and bioactive metabolites such as receptor antagonists (Silva et al., unpublished data) argues that this poorly researched environment is a propitious one in which to search for novel, exploitable microorganisms, activities, and products.

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