Microbes Environ. Vol. 20, No. 2, 110–116, 2005
http://wwwsoc.nii.ac.jp/jsme2/

Isolation and Identification of Bacterial Strains Producing Diffusible Growth Factor(s) for *Catellibacterium nectariphilum* strain AST4^T

YASUHIRO TANAKA[1,2], SATOSHI HANADA[1]*, HIDEYUKI TAMAKI[1], KAZUNORI NAKAMURA[1] and YOICHI KAMAGATA[1]

[1] *Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), Central 6, 1–1–1 Higashi, Tsukuba, Ibaraki 305–8566, Japan*
[2] *Department of Research Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, 4–3–11 Takeda, Kofu, Yamanashi 400–8511, Japan*

(Received March 15, 2005—Accepted April 4, 2005)

Catellibacterium nectariphilum was recently proposed as a new genus and species within the '*Rhodobacter* group' in the α-3 subgroup of *Proteobacteria*. This species did not show significant growth on a nutrient medium, and required unknown growth factor(s) for vigorous growth. The growth factor(s) would be diffused in the supernatant of the enrichment culture of the activated sludge from which *C. nectariphilum* was isolated. We have searched for microorganisms that produce growth factor(s) for *C. nectariphilum*, and obtained three bacterial strains, designated GF9, GF20 and GF22, from the same sludge. A phylogenetic analysis based on the 16S rRNA gene sequence indicated that these strains were closely related to each other, and all belonged to the genus *Sphingomonas*. Addition of a supernatant prepared from a culture of strain GF9 clearly increased the cell yield and growth rate of *C. nectariphilum*. Growth-promoting activity in other species of the family *Sphingomonadaceae* was also measured. The results indicated that various *Sphingomonas* species and their relatives had a similar ability and it was widely distributed within the family *Sphingomonadaceae*. The growth factor(s) produced by *Sphingomonas* species have not been identified, but were heat-stable, non-peptides and low-molecular weight (below 1,000 Da) compound(s).

Key words: growth promotion, activated sludge, *Catellibacterium nectariphilum*, *Sphingomonas*

Microbial community analyses using molecular biological techniques have suggested that most of the bacteria, possibly 90–99%, in natural and artificial environments remain unknown and have yet to be cultivated. Quite a few microbiologists believe that these unknowns can not be cultivated using conventional methods[1,26]. One reason for this would be a lack of growth factor(s) in the artificial isolation media used. Actually, some bacteria can not grow in artificial medium alone since they require materials supplied by neighboring bacteria[8,19,20]. Recently, it was reported that the addition of acylated homoserine lactone, a signal compound used in bacterial quorum-sensing, to isolation media increased numbers of cultivated cells (most-probable-number) when lake and sea water samples were used as a source[3,4,6]. Transfers of growth-promoting materials between heterogeneous bacterial species, *e.g., Wolinella recta* and *Bacteroides* species, *Bacteroides forsythus* and *Fusobacterium necleatum*, have been observed in the human oral cavity[7,27]. A similar promotion of growth by the following materials was also observed: 2-amino-3-carboxy-1,4-naphtoquinone and 1,4-dihydroxy-2-naphtoic acid produced by *Propionibacterium freudenreichii* specifically enhanced the growth of bifidobacteria such as *Bifidobacterium longum* and *Bifidobacterium breve*[13,18]. These findings suggest to us that bacteria yet to be cultured could be enriched by adding the supernatant of other bacterial cultures, since the supernatant possibly contains unknown growth factors. So we attempted

* Corresponding author; E-mail: s-hanada@aist.go.jp, Tel: +81–29–861–6591, Fax: +81–29–861–6587

to obtain yet to be cultured bacteria using isolation media supplemented with the supernatant of another bacterial culture. We succeeded in isolating a bacterial strain requiring a diffusible compound(s) produced by other bacteria for vigorous growth. An isolate designated strain AST4^T was obtained from an activated sludge[25]. On the basis of phenotypic and phylogenetic data, we propose a new genus and species within the '*Rhodobacter* group' in the α-3 subgroup of *Proteobacteria*, with the name *Catellibacterium nectariphilum*[25].

Although *C. nectariphilum* showed little growth in a medium containing tryptone peptone, yeast extract and D-glucose, its growth (growth rate and cell yield) was vigorous when the supernatant from an enrichment culture of whole activated sludge was added to the medium[25]. This certainly indicated that something in the activated sludge caused the vigorous growth of *C. nectariphilum*. It was also found that a bacterial strain affiliated with the genus *Sphingomonas* markedly promoted the growth of strain AST4^T [25]. However, the procedure used to isolate the growth-promoting strain was not mentioned at all in the previous report. Furthermore, it has not been elucidated which kinds of bacteria in the sludge culture provided the growth factor(s) for *C. nectariphilum*. In this paper, we describe newly isolated strains that supply *C. nectariphilum* with growth factor(s), and the biochemical and physical properties of the growth factor(s) produced by such a bacterium.

Materials and Methods

Culture conditions

NPB medium (pH 7.0) containing 10.0 g of tryptone peptone (Difco, Detroit, USA), 2.0 g of yeast extract (Difco), 1.0 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of K_2HPO_4, 0.5 g of KH_2PO_4 and 5.0 g of D-glucose in 1,000 ml of distilled water was used as a basal medium in this study. To cultivate *C. nectariphilum* strain AST4^T (NBRC 100046^T=JCM 11959^T=DSM 15620^T), NPB medium supplemented with 10% (v/v) supernatant from an enrichment culture of whole activated sludge was used. This enrichment culture was prepared as follows: 50 μg (wet weight) of activated sludge sample (the same sludge from which strain AST4^T was isolated) was inoculated into 100 ml of NPB medium, and incubated for 4 days at 30°C with shaking (200 rpm).

The following authentic bacteria used as references were cultured in NPB medium at 30°C: *Sphingomonas sanguinis* IAM 12578^T, *Sphingomonas mali* NBRC 15500^T, *Sphingobium yanoikuyae* NBRC 15102^T, *Sphingobium herbicidovorans* NBRC 16415^T, *Sphingobium chlorophenolica* NBRC 16172^T, *Novosphingobium rosa* IAM 14222^T, *Novosphingobium capsulata* NBRC 12533^T, *Sphingopyxis macrogoltabida* NBRC 15033^T, *Sphingopyxis terrae* NBRC 15098^T, *Sphingopyxis* sp. NBRC 15915, *Escherichia coli* DH5α (Takara), *Pseudomonas putida* IAM 1049 and *Bacillus subtilis* IAM 12118^T.

Isolation of strains producing growth factor(s) for C. nectariphilum *strain AST4^T*

A portion of the activated sludge from which *C. nectariphilum* strain AST4^T was isolated was inoculated onto a plate of NPB agar, and incubated for 4 days at 30°C. Thirty strains, named GF1 through 30, emerged as single colonies on the plate and were successfully isolated.

To test its effect on the growth of *C. nectariphilum*, each strain was individually grown in 5 ml of NPB medium at 30°C, and a supernatant was prepared from stationary-grown cultures (optical density at 660 nm [OD_{660}] of 1.9 to 5.3) by centrifugation (33,768×*g* for 20 min). The supernatant was added to NPB medium at a final concentration of 10% (v/v) after autoclaving. Each supernatant-containing medium was inoculated with *C. nectariphilum*, and incubated at 30°C for 2 days. The growth rate and cell yield of *C. nectariphilum* in each supernatant-containing culture was estimated by measuring cell density (OD_{660}), and compared to that in NPB medium without the supernatant. The growth of *C. nectariphilum* was notably enhanced by the supernatant prepared from an enrichment culture of whole activated sludge[25], and near full growth was attained in 2 days. The cell density of the 2-day culture was approx. 7 times greater than that of the supernatant-free culture. Thus we regarded an isolate to have effective growth-promoting activity, when the addition of its supernatant enhanced the cell yield of *C. nectariphilum* by 7-fold or more.

Reference species such as *E. coli, P. putida, B. subtilis* and authentic *Sphingomonadaceae* species were also tested for their ability to promote the growth of *C. nectariphilum* strain AST4^T under the same conditions.

Phylogenetic analysis of GF strains based on the 16S rRNA gene sequence

16S rRNA gene sequences of isolated GF strains were obtained by PCR using two oligonucleotide primers, 5'-GTGCCAGCMGCCGCGG-3'[16] and 5'-ACGGYTACCTTGTTACGACTT-3'[14] (corresponding to positions 530–545 and 1492–1512 of the *E. coli* 16S rRNA, respectively). Partial nucleotide fragments of the 16S rRNA gene (corresponding to position 912–1389 of the *E. coli* 16S rRNA) in the strains were sequenced by the dideoxynucleotide chain-termination

method using a BigDye terminator cycle sequencing FS ready reaction kit (Applied Biosystems, Lincoln Centre Drive Foster, CA, USA). The 16S rRNA gene sequences were compared to those in the NCBI database using the standard nucleotide-nucleotide homology search program (BLAST; http://www.ncbi.nlm.nih.gov/BLAST/). The nucleotide sequence data determined in this study appear in the DDBJ/EMBL/GenBank databases with the accession numbers AB101544 to AB101573.

Characterization of growth factor(s) for C. nectariphilum

Strain GF9, one of the most effective growth factor-producers, was used to investigate when the growth factor(s) was effused into a medium. A portion of strain GF9 culture was collected at periodic intervals (12, 18, 24, 36, 48, 60 and 85 h). The collected cultures were centrifuged at 33,768×*g* for 20 min, and the supernatant was subjected to a bioassay after autoclaving.

The molecular weight of the growth factor(s) produced by strain GF9 was estimated with ultrafiltration devices, Microcon YM-50, Microcon YM-10, Microcon YM-3 (molecular cut off, 50,000, 10,000 and 3,000, respectively; Millipore, Billerica, MA, USA) and Microsep 1K (molecular cut off, 1,000; Nihon Pall, Tokyo, Japan).

Tolerance of protease or alkaline- and acid-treatments was also studied with the supernatant from a fully grown culture of strain GF9. PRONASE protease from *Streptomyces griseus* (10 mg/ml in 100 mM potassium phosphate buffer [pH 7.0]; Wako, Osaka, Japan) was added to the supernatant at a concentration of 0.2 mg/ml, and incubated at 37°C for over 2 h. The reaction mixture was autoclaved, and residual growth-promoting activity for *C. nectariphilum* was evaluated. Alkaline- and acid-treatments were performed as follows: the pH of the supernatant was adjusted to 2.0 and 10.0 with 6N HCl and 6N NaOH, respectively. After incubation at room temperature for 6 h, the supernatants were neutralized and autoclaved, and the growth-promoting activity was measured.

Results and Discussion

Isolation of bacterial strains producing growth factor(s) for C. nectariphilum *strain AST4^T*

C. nectariphilum strain AST4^T was recently reported to require some diffusible growth factor(s) produced by other microorganisms for vigorous growth[25]. This strain did not show significant growth on a standard nutrient medium such as NPB medium, but its growth was clearly enhanced on the addition of supernatant (10%, v/v) from an enrichment culture of the whole activated sludge. This indicated that the activated sludge contained bacteria producing the growth factor(s) for strain AST4^T. To determine the growth factor-producers, we first isolated thirty bacterial strains, designated GF1 through 30, from the activated sludge using NPB agar medium. The phylogenetic relationships of these isolates were determined based on the analysis of partial 16S rRNA gene sequences (Table 1). Almost all isolates (29 strains) belonged to the phylum *Proteobacteria* while one, GF25, was akin to the *Actinobacteria* (formally Gram-positive bacteria with a high G+C content). Each GF strain was incubated in NPB medium, and a supernatant was obtained from each culture to test whether it can enhance the cell yield of strain AST4^T. In three of the thirty isolates, i.e.

Table 1. Phylogenetic affiliations based on partial 16S rRNA gene sequences among strains isolated from the activated sludge (GF strains)

Isolated strains[a]	Most related authentic strains (Accession number)	Taxonomic affiliation[b]	Similarity (%)
GF1/GF4/GF6/GF7/GF8/GF11/GF13/GF18/GF26/GF28	*Brachymonas denitrificans*[11] (D14320)	β	99.8
GF2/GF3/GF5/GF10/GF12/GF14/GF15/GF16/GF17/GF30	*Paracoccus marcusii*[9] (Y12703)	α	94.3
GF9/GF20/GF22	*Sphingomonas adhaesiva*[24] (D13722)	α	99.8
GF19/GF29	*Rhodobacter veldkampii*[12] (D16421)	α	96.7
GF21	*Ochrobactrum intermedium*[17] (AJ242583)	α	98.5
GF23	*Ramlibacter henchirensis*[10] (AF439400)	β	97.7
GF24	*Thermomonas haemolytica*[5] (AJ300185)	γ	98.3
GF25	*Leucobacter komagatae*[22] (D17751)	High G+C	97.9
GF27	*Zoogloea ramigera*[21] (D14255)	β	99.2

[a] Strains with identical 16S rRNA gene sequences are indicated together. [b] α, β and γ indicate α-, β- and γ-*Proteobacteria*, respectively. High G+C indicates Gram-positive bacteria with a high G+C DNA content (=the phylum *Actinobacteria*).

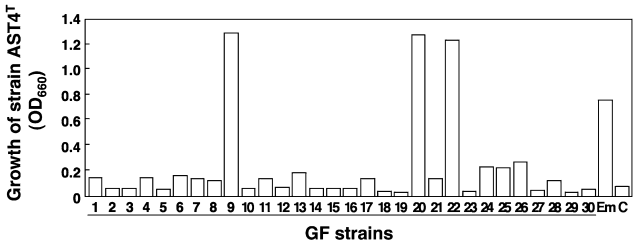

Fig. 1. Effects of culture supernatants from GF strains on the growth of *C. nectariphilum* strain AST4^T. The numbers 1–30 indicate strains GF1–30; Em, enrichment of the whole activated sludge; C, control (NPB medium without any supernatant). *C. nectariphilum* strain AST4^T was cultivated in NPB medium containing 10% (v/v) supernatant prepared from each isolated strain and the whole activated sludge at 30°C for 2 days. The growth was shown by the cell density at 660 nm.

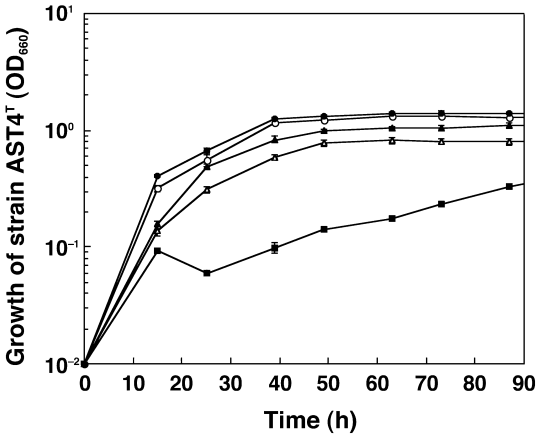

Fig. 2. Growth of *C. nectariphilum* strain AST4^T on NPB medium containing supernatant from strain GF9 at various concentrations. The growth of strain AST4^T in the medium containing 0, 0.5, 1.0, 5.0 and 10.0% (v/v) autoclaved supernatant is described by ■, △, ▲, ○ and ●, respectively. The error bars indicate standard deviations (n=3).

strains GF9, GF20 and GF22, effective growth-promoting activity was clearly found (Fig. 1). The phylogenetic analysis based on partial 16S rRNA gene sequences indicated that these three strains had the same 16S rRNA gene sequence, and were closely related to *Sphingomonas adhaesiva*[23] with high (99.8%) sequence similarity (Table 1). Based on the phylogenetic analysis, these three isolates were considered to be the same species in the genus *Sphingomonas*. The ability of each strain to promote growth was more effective than that shown by the supernatant of the whole activated sludge culture. Thus, it is quite likely that the *Sphingomonas* strain played the leading role in the ability of the activated sludge to promote the growth of *C. nectariphilum* strain AST4^T.

Growth promotion of C. nectariphilum *strain AST4^T by supernatant from strain GF9*

Figure 2 shows growth curves of *C. nectariphilum* AST4^T in NPB medium containing 0 to 10% (v/v) supernatant obtained from the culture of strain GF9, one of the newly isolated growth factor-producers. The rate of growth of *C. nectariphilum* strain AST4^T was enhanced in proportion to the concentration of supernatant added. The doubling time of strain AST4^T in the logarithmic phase in NPB medium containing 10% (v/v) supernatant was calculated to be 14.5 h, while that in NPB medium without the supernatant was 30.2 h. All cultures containing supernatant reached a stationary phase after 2 days. The cell density (OD_{660}) of strain AST4^T was approx. 10 times greater in NPB medium containing 10% supernatant than in the supernatant-free medium after 2 days of culture. Even in the NPB medium that contained 0.5% (v/v) supernatant, an approx. 6-fold increase in cell density was observed. The culture without supernatant grew slowly, and reached a stationary phase after 5 days. However, the cell density (OD_{660}) of the culture collected at the stationary phase did not exceed 0.5. These results indicated that some diffusive compound(s) produced by strain GF9 enhanced both the cell yield and growth rate of *C. nectariphilum* strain AST4^T.

Figure 3 shows the ability to promote the proliferation of *C. nectariphilum* strain AST4^T in each phase of growth. A portion of the GF9 culture was collected at regular intervals (12, 18, 24, 36, 48, 60 and 85 h) and the growth-promoting activity in the supernatants was measured. The activity was detected even in the early logarithmic phase (12 h) and increased during the growth phase of *Sphingomonas* sp. GF9. The level of activity peaked in the early stationary phase, and decreased slightly in the late stationary phase. The results suggested that strain GF9 constantly produced growth factor(s) throughout its life cycle, and the production stopped as the growth stagnated.

Effects of supernatants from strains in the family Sphingomonadaceae *and other bacteria on the growth of strain AST4^T*

The phylogenetic analysis based on the 16S rRNA gene sequence suggested that growth factor(s)-producers isolated from the activated sludge, i.e., strains GF9, GF20 and GF22, should be classified into the genus *Sphingomonas* as described above. The next question to answer is whether the ability to promote the growth of *C. nectariphilum* strain AST4^T is limited to our isolates, or evenly distributed

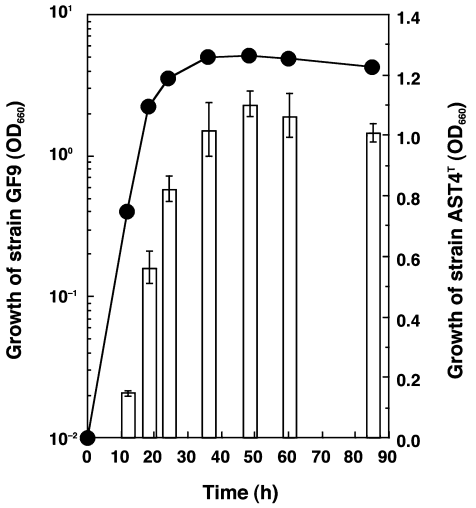

Fig. 3. Production of growth-promoting compound(s) during the cultivation of strain GF9. The growth of strain GF9 is indicated by ●. White columns indicate the cell density (OD_{660}) of the cultures of *C. nectariphilum* strain AST4^T incubated in NPB medium containing 10% (v/v) supernatant of GF9 culture collected at regular intervals. Each incubation was done at 30°C for 2 days. The error bars indicate standard deviations (*n*=3).

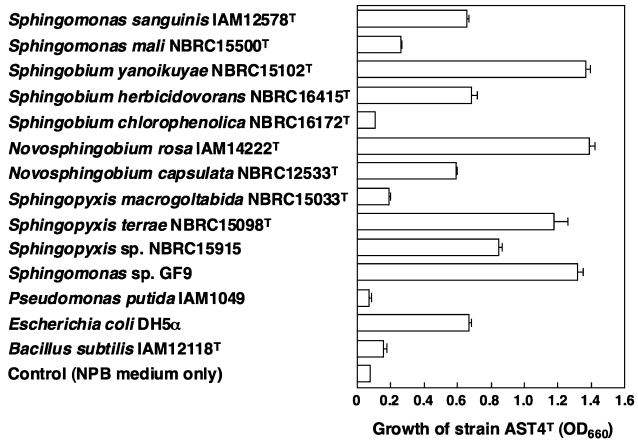

Fig. 4. Growth-promoting effects of supernatants from various *Sphingomonadaceae* species, *P. putida* IAM 1049, *E. coli* DH5α and *B. subtilis* IAM 12118^T on the growth of *C. nectariphilum* strain AST4^T. *C. nectariphilum* strain AST4^T was cultivated in supernatant-containing (10%, v/v) medium at 30°C for 2 days. The error bars indicate standard deviations (*n*=3).

among other *Sphingomonas* species and related bacteria. Therefore, we measured the growth-promoting activity of supernatants from other species belonging to *Sphingomonas* and related *Sphingomonadaceae* genera, *Novosphingobium*, *Sphingobium* and *Sphingopyxis*. In addition, other unrelated bacterial species, *Pseudomonas putida* IAM 1049, *Escherichia coli* DH5α and *Bacillus subtilis* IAM 12118^T, were also tested for their effect on the growth of strain AST4^T (Fig. 3).

The ability to promote the growth of *C. nectariphilum* strain AST4^T was found in almost all species in the family *Sphingomonadaceae*. Notably, the supernatants obtained from cultures of *S. yanoikuyae* NBRC 15102^T, *N. rosa* IAM 14222^T and *S. terrae* NBRC 15098^T had growth-promoting activity equal to that of strain GF9. Although a similar effect was obtained with the supernatant of the *E. coli* culture, it was just half of that with strain GF9. The other reference species, *Bacillus subtilis* and *Pseudomonas putida*, had no effect. In the former experiments on growth promotion by GF strains (Fig. 1), the isolates other than *Sphingomonas*-related strains did not have a significant promotional effect on the growth of *C. nectariphilum* (Fig. 1). Table 1 shows that the strains having no effective growth-promoting activity were phylogenetically diverse and widely distributed among the *Proteobacteria* and *Actinobacteria* (each was closely related to the following authentic species: *Brachymonas denitrificans*; *Paracoccus marcusii*; *Rhodobacter veldkampii*; *Ochrobactrum intermedium*; *Ramlibacter henchirensis*; *Thermomonas haemolytica*; *Leucobacter komagatae*; *Zoogloea ramigera*, respectively). These results suggested that the ability to produce the compound(s) promoting the growth of strain AST4^T is not ubiquitous to all bacteria but often found in species belonging to the family *Sphingomonadaceae*.

Growth factor(s) produced by strain GF9

To determine the molecular weight of the growth-promoting compound(s), the supernatant of strain GF9 was treated with ultrafiltration devices. The compound(s) passed through all the filters used in this study (molecular cut off, 50,000, 10,000, 3,000 and 1,000) and enhanced the growth of *C. nectariphilum* strain AST4^T. Therefore, the growth-promoting compound(s) would have a low molecular weight (below 1,000 Da). No reduction in the promotion of growth was observed using the supernatants treated with protease (PRONASE protease from *Streptomyces griseus*), and the activity was entirely maintained after alkaline- (pH 10.0) and acid- (pH 2.0) treatments. The compound(s) were heat-stable, and not deactivated by autoclaving. These findings suggest that the growth-promoting compound(s) were not peptides or other labile materials.

Vitamins, fatty acids and carbohydrates often serve as growth factor(s) for microorganisms in microbial communities[22]. We examined whether the growth of *C. nectariphilum* strain AST4^T was promoted by various vita-

mins, short-chained fatty acids, sugars, amino acids and alcohols commonly used in biological laboratories, but found no growth-enhancing effects. Recently, several non-peptide chemicals, norepinephrine, dopamine, isoprotenol, 5-hydroxytriptamine, 1,4-dihydroxy-2-naphthoic acid, *N*-(oxohexanoyl)-DL-homoeserine lactone, *N*-(butyryl)-DL-homoserine lactone and cyclic AMP have been reported to promote the growth of some kinds of bacteria[2–4,6,13,15]. However, none of these compounds enhanced the growth of *C. nectariphilum* strain AST4^T. The growth-promoting compound(s) for strain AST4^T are, therefore, clearly different from known growth-promoting or -stimulating compound(s). The compound(s) produced by strain GF9 have yet to be identified, but their isolation and structural analyses are now in progress.

Although it has not been clarified whether the compound(s) can enhance the growth of any other bacteria in standard nutrient media, the possibility seems likely. Identification of the growth-promoting compound(s) would be important to establish a method of culturing uncultivable bacteria requiring some compound(s) produced by other microorganisms.

Acknowledgements

This study was carried out as a part of the project for NEDO Industrial Technology Researcher at the National Institute of AIST.

References

1) Amann, R.I., W. Ludwig and K.H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev. **59**: 143–169.
2) Belay, T., H. Aviles, M. Vance, K. Fountain and G. Sonnenfeld. 2003. Catecholamines and in vitro growth of pathogenic bacteria: enhancement of growth varies greatly among bacterial species. Life Sci. **73**: 1527–1535.
3) Bruns, A., H. Cypionka and J. Overmann. 2002. Cyclic AMP and acyl homoserine lactones increase the cultivation efficiency of heterotrophic bacteria from the Central Baltic Sea. Appl. Environ. Microbiol. **68**: 3978–3987.
4) Bruns, A., U. Nubel, H. Cypionka and J. Overmann. 2003. Effect of signal compounds and incubation conditions on the culturability of freshwater bacterioplankton. Appl. Environ. Microbiol. **69**: 1980–1989.
5) Busse, H.J., P. Kampfer, E.R. Moore, J. Nuutinen, I.V. Tsitko, E.B. Denner, L. Vauterin, M. Valens, R. Rossello-Mora and M.S. Salkinoja-Salonen. 2002. *Thermomonas haemolytica* gen. nov., sp. nov., a gamma-proteobacterium from kaolin slurry. Int. J. Syst. Evol. Microbiol. **52**: 473–483.
6) Bussmann, I., B. Philipp and B. Schink. 2001. Factors influencing the cultivability of lake water bacteria. J. Microbiol. Methods **47**: 41–50.
7) Grenier, D. and D. Mayrand. 1986. Nutritional relationships between oral bacteria. Infect. Immun. **53**: 616–620.
8) Guan, L.L., H. Onuki and K. Kamino. 2000. Bacterial growth stimulation with exogenous siderophore and synthetic *N*-acyl homoserine lactone autoinducers under iron-limited and low-nutrient conditions. Appl. Environ. Microbiol. **66**: 2797–2803.
9) Harker, M., J. Hirschberg and A. Oren. 1998. *Paracoccus marcusii* sp. nov., an orange gram-negative coccus. Int. J. Syst. Bacteriol. **48**: 543–548.
10) Heulin, T., M. Barakat, R. Christen, M. Lesourd, L. Sutra, G. De Luca and W. Achouak. 2003. *Ramlibacter tataouinensis* gen. nov., sp. nov., and *Ramlibacter henchirensis* sp. nov., cyst-producing bacteria isolated from subdesert soil in Tunisia. Int. J. Syst. Evol. Microbiol. **53**: 589–594.
11) Hiraishi, A., Y. Shin and J. Sugiyama. 1995. *Brachymonas denitrificans* gen. nov., sp. nov., an aerobic chemoorganotrophic bacterium which contains rhodoquinones, and evolutionary relationships of rhodoquinone producers to bacterial species with various quinone classes. J. Gen. Appl. Microbiol. **41**: 99–117.
12) Hiraishi, A. and Y. Ueda. 1994. Intragenetic structure of the genus *Rhodobacter*; Transfer of *Rhodobacter sulfidophilus* and related marine species to the genus *Rhodovulum* gen. nov. Int. J. Syst. Bacteriol. **44**: 15–23.
13) Isawa, K., K. Hojo, N. Yoda, T. Kamiyama, S. Makino, M. Saito, H. Sugano, C. Mizoguchi, S. Kurama, M. Shibasaki, N. Endo and Y. Sato. 2002. Isolation and identification of a new bifidogenic growth stimulator produced by *Propionibacterium freudenreichii* ET-3. Biosci. Biotechnol. Biochem. **66**: 679–681.
14) Kane, M.D., L.K. Poulsen and D.A. Stahl. 1993. Monitoring the enrichment and isolation of sulfate-reducing bacteria by using oligonucleotide hybridization probes designed from environmentally derived 16S rRNA sequences. Appl. Environ. Microbiol. **59**: 682–686.
15) Kinney, K.S., C.E. Austin, D.S. Morton and G. Sonnenfeld. 1999. Catecholamine enhancement of *Aeromonas hydrophila* growth. Microb. Pathog. **26**: 85–91.
16) Lane, D.J. 1991. 16S/23S rRNA sequencing, pp. 115–175. *In* E. Stackebrandt and Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. Wiley-Interscience, West Sussex, England.
17) Lebuhn, M., W. Achouak, M. Schloter, O. Berge, H. Meier, M. Barakat, A. Hartmann and T. Heulin. 2000. Taxonomic characterization of *Ochrobactrum* sp. isolates from soil samples and wheat roots, and description of *Ochrobactrum tritici* sp. nov. and *Ochrobactrum grignonense* sp. nov. Int. J. Syst. Evol. Microbiol. **50**: 2207–2223.
18) Mori, H., Y. Sato, N. Taketomo, T, Kamiyama, Y. Yoshiyama, S. Meguro, H. Sato and T. Kaneko. 1997. Isolation and structural identification of bifidogenic growth stimulator produced by *Propionibacterium freudenreichii*. J. Dairy. Sci. **80**: 1959–1964.
19) Ohno, M., I. Okano, T. Watsuji, T. Kakinuma, K. Ueda and T. Beppu. 1999. Establishing the independent culture of a strictly symbiotic bacterium *Symbiobacterium thermophilum* from its supporting *Bacillus* strain. Biosci. Biotechnol. Biochem. **63**: 1083–1090.
20) Rhee, S.-K., C.O. Jeon, J.-W. Bae, K. Kim, J.J. Song, J.-J. Kim, S.-G. Lee, H.-I. Kim, S.-P. Hong, Y.-H. Choi, S.-M. Kim and M.-H. Sung. 2002. Characterization of *Symbiobacterium toebii*, an obligate commensal thermophile isolated from compost. Extre-

mophiles **6**: 57–64.
21) Shin, Y.K., A. Hiraishi and J. Sugiyama. 1993. Molecular systematics of the genus *Zoogloea* and emendation of the genus. Int. J. Syst. Bacteriol. **43**: 826–831.
22) Slater, B.H. 1981. Mixed cultures and microbial communities, pp. 1–24. *In* Bushell and Slater (ed.), Mixed culture fermentations, Academic Press, New York.
23) Takeuchi, M., H. Sawada, H. Oyaizu and A. Yokota. 1994. Phylogenetic evidence for *Sphingomonas* and *Rhizomonas* as nonphotosynthetic members of the alpha-4 subclass of the *Proteobacteria*. Int. J. Syst. Bacteriol. **44**: 308–314.
24) Takeuchi, M., N. Weiss, P. Schumann and A. Yokota. 1996. *Leucobacter komagatae* gen. nov., sp. nov., a new aerobic gram-positive, nonsporulating rod with 2,4-diaminobutyric acid in the cell wall. Int. J. Syst. Bacteriol. **46**: 967–971.
25) Tanaka, Y., S. Hanada, A. Manome, T. Tsuchida, R. Kurane, K. Nakamura and Y. Kamagata. 2004. *Catellibacterium nectariphilum* gen. nov., sp. nov., which requires a diffusible compound from a strain related to the genus *Sphingomonas* for vigorous growth stimulation. Int. J. Syst. Evol. Microbiol. **54**: 955–959.
26) Wagner, M., R. Amann, H. Lemmer and K.-H. Schleifer. 1993. Probing activated sludge with origonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. Appl. Environ. Microbiol. **59**: 1520–1525.
27) Wyss, C. 1989. Dependence of proliferation of *Bacteroides forsythus* on exogenous *N*-acetylmuramic acid. Infect. Immun. **57**: 1757–1759.