JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 89, No. 5, 469–473. 2000

Rapid Detection of *Nocardia amarae* in the Activated Sludge Process Using Enzyme-Linked Immunosorbent Assay (ELISA)

KEISUKE IWAHORI,* NAOYUKI MIYATA, SACHIKO MORISADA, AND NORIAKI SUZUKI§

Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan

Received 24 December 1999/Accepted 18 February 2000

***Nocardia amarae*, a mycolic acid-containing bacterium, has often been reported to cause foaming of activated sludge in wastewater treatment plants. In this study, the number of *N. amarae* cells in the activated sludge process was estimated by enzyme-linked immunosorbent assay (ELISA) with anti-*N. amarae* polyclonal antibody. Use of the antibody enabled *N. amarae* to be detected at levels of 10^4 to 10^7 colony forming units. On the other hand, the antibody reacted with only a small portion of activated sludge, in which no *N. amarae* cells were detected by the plate count method. Competitive ELISA was employed to estimate the *N. amarae* cells in samples taken from a municipal wastewater treatment plant, including raw wastewater and activated sludge foam. The cell numbers estimated by competitive ELISA corresponded well with those obtained by plate counts. Hence, the antibody produced in this study was shown to be effective for the rapid monitoring of *N. amarae* in the activated sludge process.**

[**Key words:** scum, foaming, *Nocardia* (*Gordona*) *amarae*, activated sludge, enzyme-linked immunosorbent assay]

The activated sludge process has contributed widely to the treatment of domestic and industrial wastewater. One of the most important and well-known characteristics of this process is solid-liquid separation. However, the formation of a highly viscous and stable foam is often reported in treatment plants worldwide (1), giving rise to operational problems such as effluent quality deterioration, increased time requirements for plant maintenance, the creation of malodors by decomposing foam, and in extreme cases, hazardous working conditions resulting from foam spilling out of the aeration basin. Aside from these operational inconveniences, it is very important to solve the foam problem from a sanitary point of view, since a novel foam-producing bacterium isolated from activated sludge plants has been identified as an opportunistic pathogen (2).

Nocardia amarae (3) [presently *Gordona amarae* (4, 5)], which has often been isolated as the dominant microorganism from foaming activated sludge, belongs to a group of mycolic acid-containing bacteria that includes actinomycetes such as *Rhodococcus* spp. (6) and *Mycobacterium* sp. (7). The involvement of *N. amarae* in foaming in the activated sludge process has been extensively investigated from the following viewpoints: nutritional requirements (6), cell surface hydrophobicity and affinity of the cells to air bubbles (8), and the production of surface-active agents (9).

For the purpose of controlling the growth of the microorganism in aeration tanks, relatively quick and practical methods for monitoring *N. amarae* have been developed using the plate count technique to enumerate viable cells (10, 11). However, cultivation for several days is needed for the confirmation of viable colonies on the plate. In view of this, the development of more rapid means of *N. amarae* detection is desired.

Recently, two molecular approaches have been introduced as useful tools for understanding the population dynamics of *N. amarae* in the activated sludge process. rRNA-targeted oligonucleotide hybridization probes were investigated by de los Reyes *et al.* (12, 13), who reported that the use of such probes specific for *N. amarae* strains led to the detection of the targeted microorganisms and their accurate quantification in activated sludge. The other approach, an immunological method using fluorescently labeled antibody, was applied by Hernandez *et al.* (14) to the enumeration of *N. amarae* in activated sludge and anaerobic digesters. In their study, the filamentous cells of *N. amarae* were shown to be stained with the fluorescent antibody, and the cell mass could be detected by measuring the filament length.

Although both the above approaches have provided many useful findings on bacterial population dynamics, the immunological method appears more suitable as a useful means of rapidly monitoring the targeted bacterium on site, *i.e.* in wastewater treatment plants. Therefore, in the present study the novel, rapid and accurate detection of *N. amarae* by enzyme-linked immunosorbent assay (ELISA) using anti-*N. amarae* polyclonal antibody was experimentally investigated. In addition, the applicability of ELISA for effective monitoring of *N. amarae* in activated sludge is discussed from a practical point of view.

MATERIALS AND METHODS

Bacterial strains and growth conditions The bacterial strains used in this study are listed in Table 1. *N. amarae* was previously isolated from activated sludge scum and characterized by Sakai *et al* (15). The other strains were obtained from the American Type Culture Collection (ATCC) or the Institute for the Fermentation, Osaka (IFO). All the strains were grown in the liquid media shown in Table 1 at 28°C. MS medium consisted of 5.0 g peptone, 2.5 g yeast extract, 1.0 g glucose and 4.5 g Na propionate per liter; pH 7.5. Media 227, 297, and 802 were prepared according to the IFO microorganisms

* Corresponding author.
§ Present address: Bridgestone Elastic Co. Ltd., 4560 Chihama, Daito-cho, Ogasa-gun, Shizuoka 437-1493, Japan.

TABLE 1. Bacterial strains and growth media used in the study

Organism	Abbreviation	Medium
Nocardia amarae	NA	MS
Gordona amarae IFO 15530	GA	227
Nocardia nova IFO 15556	NN	227
Dietzia maris IFO 15801	DM	297
Rhodococcus equi IFO 14956	REq	802
Rhodococcus erythropolis ATCC 4277	REr	MS
Rhodococcus rhodochrous ATCC 13808	RRh	MS
Rhodococcus ruber IFO 15591	RRu	227
Mycobacterium phlei IFO 13160	MP	802
Mycobacterium smegmatis IFO 13167	MS	802
Tsukamurella paurometabolum IFO 12160	TP	802
Corynebacterium variabilis IFO 15286	CV	802

catalog (16). Cells were harvested in the late logarithmic growth phase, washed with sterilized water, and normally stored at −80°C after freeze-drying. However, in the experiments for counting the *N. amarae* cell numbers, harvested cells were used immediately.

Activated sludge samples Activated sludge acclimated to artificial sewage wastewater by fill-and-draw cultivation (17) was used to the test of specificity of anti-*N. amarae* antibody. The sludge was collected and washed by centrifugation at 800 × *g* for 5 min.

The following samples were obtained from a municipal sewage treatment plant (Fig. 1): influent (raw sewage), effluent from the primary settling tank (primary effluent), effluent from the aeration basin, scum in the secondary clarifier, and effluent from the secondary clarifier (secondary effluent). In a previous study, *N. amarae* was shown to be a causative bacterium of foaming in this plant (10). The mixed liquor suspended solid (MLSS) and water content were determined according to the standard methods for the examination of sewage (18).

Anti-*N. amarae* antibody Freeze-dried *N. amarae* cells were suspended in physiological saline and dispersed by sonication (130 W, 1 min), resuspended in 10% formalin solution, and allowed to stand at 37°C for 24 h in order to kill the cells. A portion of the killed cells (dry weight, 200 μg) was subcutaneously injected into three New Zealand White rabbits (nos. 1 to 3) at 2-week intervals over 6 weeks. All the sera from immunized rabbits 1 and 2 were harvested 50 d after the first immunization. In the case of rabbit 3, all the serum was harvested after 70 d.

ELISA ELISA was carried out using a 96-well microtiter plate. *N. amarae* cells were diluted with phosphate buffer saline (PBS) to a concentration of 5 μg (dry weight)/ml, and 200 μl of the suspension was put into each well. Cells were precipitated by centrifugation (600 × *g*, 20 min) and immobilized by treatment with 0.1% glutaraldehyde for 30 min at room temperature (19). After removing excess glutaraldehyde, 250 μl of a blocking solution containing 0.1% bovine serum albumin (BSA) and 0.1 M glycine in PBS was added, and the plate was allowed to stand for 2 h at 28°C. The wells were washed with PBS containing 0.05% Tween 20 (PBST) and finally filled out with PBS containing 0.25% BSA, 3 mM $MgCl_2$, and 0.15 M NaCl. The plate-immobilized cells were stored at 4°C. Anti-*N. amarae* antiserum was diluted to 1 : 256 with PBST. A 200-μl portion of the diluted antiserum was added to each well and the plate was incubated for 2 h at 28°C. After washing thoroughly with PBST, the antibody associated with immobilized *N. amarae* cells was labeled with goat anti-rabbit IgG (H+L)-peroxidase conjugate (Wako Pure Chemical Industries, Osaka), which was diluted 1000 times with PBST before use. Color development at 490 nm was monitored with a microplate reader (EL 340; Bio-Tek Instruments, Winooski, VT, USA) for 30 min after addition of 200 μl of a citrate buffer, pH 4.8, containing (per liter) 400 mg *o*-phenylenediamine and 100 μl of 30% H_2O_2.

Competitive ELISA The reactivity of anti-*N. amarae* antibody with mycolic acid-containing bacteria was determined by competitive ELISA. Bacterial cells suspended in PBS were added (0.2 μg/well) to plate-immobilized *N. amarae* (see above). PBS not containing free cells was added to a control well. Diluted antiserum (final dilution rate, 1 : 256) was then added and the plate was incubated for 30 min at 28°C. After washing thoroughly with PBST, the amount of antibody absorbed on the *N. amarae* immobilized in the wells was measured in the same manner that was used in the ELISA procedure. The efficiency of inhibition by free cells in the liquid phase was determined on the basis of following calculation:

$$\text{Inhibition } (\%) = [(A_c - A_x)/A_c] \times 100$$

where A_x is the absorbance of the well supplemented with free cells and A_c is absorbance of the well without free cells (control).

Determination of *N. amarae* cells

CFU counts To determine the numbers of *N.*

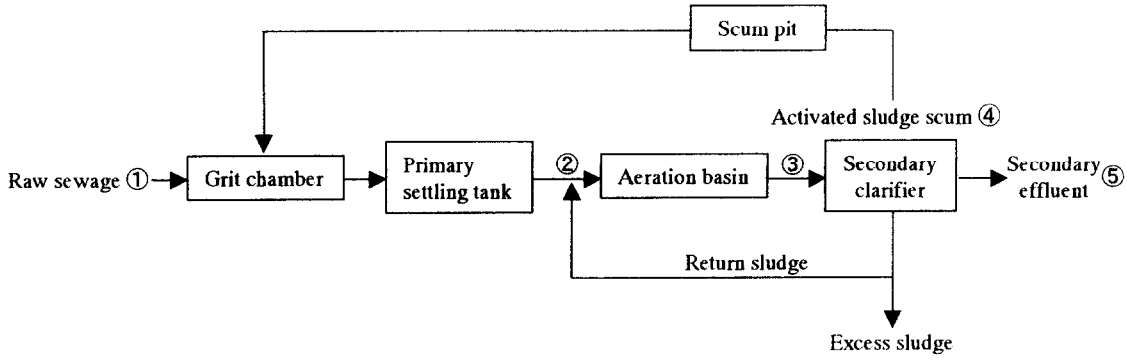

FIG. 1. Schematic diagram of the activated sludge process in the sewage treatment plant. The arrows show the streams of wastewater, effluents, and sludges. The samples used in this study were: 1, raw sewage; 2, primary effluent; 3, aeration basin effluent; 4, scum in secondary clarifier; 5, secondary effluent.

amarae cells in monocultures or in samples obtained from the activated sludge process, colony forming units (CFU) were counted using serial dilution plates of OD medium, as previously described (10). Scum was homogenized for 2 min at 10,000 rpm before use. All samples were dispersed by sonication for 8 min at 130 W before being diluted serially 10 times with Na tripolyphosphate (5 mg/*l*). *N. amarae* cell numbers are expressed as CFU per ml of a sample or per mg-MLSS of a sample.

Competitive ELISA *N. amarae* cells in samples were also determined by competitive ELISA. Samples appropriately diluted with PBS were added to plate-immobilized *N. amarae* (1 μg/well) to measure their inhibition efficiencies. *N. amarae* cells were estimated using a calibration curve (Fig. 3): *N. amarae* cells were applied to plate-immobilized *N. amarae* at 10^4 to 10^7 CFU/well to show the relationship between the *N. amarae* cell numbers and the inhibition efficiencies obtained by competitive ELISA.

RESULTS AND DISCUSSION

Production of anti-*N. amarae* polyclonal antibody In all three rabbits, the antibody titers against *N. amarae* cells increased gradually and reached maximum levels as a result of 3 or 4 immunizations. Rabbit 1 produced antiserum with the highest antibody titer (Fig. 2). On the other hand, the reactivity of the antiserum with activated sludge was much lower than that with *N. amarae* cells, especially when activated sludge was tested at concentrations of 0.5 to 1.5 μg/well (Fig. 2). This finding suggests only limited antibody reaction with other microorganisms and constituents of activated sludge. The activated sludge used in this study, which was acclimated to artificial wastewater in our laboratory, had never experienced foaming. No nocardioform bacteria were detected in the sludge by microscopic observation or with OD medium (see Materials and Methods), although the possibility of the presence of *N. amarae* and/or other related bacteria could not be definitely ruled out. The antibody from rabbit 1 was used in further experiments since it appeared to be the most useful for the specific detection of *N. amarae* in activated sludge.

Detection of *N. amarae* by competitive ELISA The competitive ELISA experiment revealed a good correlation ($r^2=0.97$) between the numbers of *N. amarae* cells and their inhibition efficiencies (Fig. 3). The anti-*N. amarae* antibody was thus able to determine CFU levels in the range from 10^4 to 10^7. In a previous study, the numbers of *N. amarae* cells in a municipal wastewater treatment plant were enumerated using the colony counting method with OD medium (20). The results, for example, 7.1×10^4, 8.5×10^5 and 4.5×10^6 CFU/mg-MLSS in raw sewage, primary effluent and sludge foam, respectively, confirmed the presence of *N. amarae* cells at levels of 10^4 to 10^6 CFU/mg-MLSS. The competitive ELISA investigated here would be able to detect such numbers of *N. amarae* cells.

Reactivity of antibody with mycolic acid-containing bacteria Mycolic acid-containing bacteria other than *N. amarae* have been reported to accumulate in activated sludge foam (7, 21, 22). To determine whether such bacteria were recognized by the antibody similarly to *N. amarae*, its cross-reactivity was tested by competitive ELISA (Fig. 4). *G. amarae* (IFO strain) exhibited a high degree of inhibition efficiency (50%), corresponding to 81% of the inhibition level of *N. amarae*. This result shows that the antibody strongly recognized *G. amarae* as it did *N. amarae*, presumably because the two strains are taxonomically identical. Except for *Rhodococcus equi, Tsukamurella paurometabolum*, and *Corynebacterium variabilis*, the antibody showed moderate recognition toward cells of the other strains, whose inhibition levels ranged from 29 to 65% that of *N. amarae*. In this study, we could not find a clear tendency in the reactivity of antibody in relation to each genus, although the results can be summarized as follows: *Nocardia* and *Gordona*≥*Dietzia, Rhodococcus* and *Mycobacterium*≥*Tsukamurella*≥*Corynebacterium*.

In treatment plants where mycolic acid-containing bacteria other than *N. amarae* predominate in the foam, it may be difficult to detect only *N. amarae* cells by the method described here due to reactivity of the antibody with the other bacteria. However, since *N. amarae* has been shown to be dominant in most plants (15, 23), the

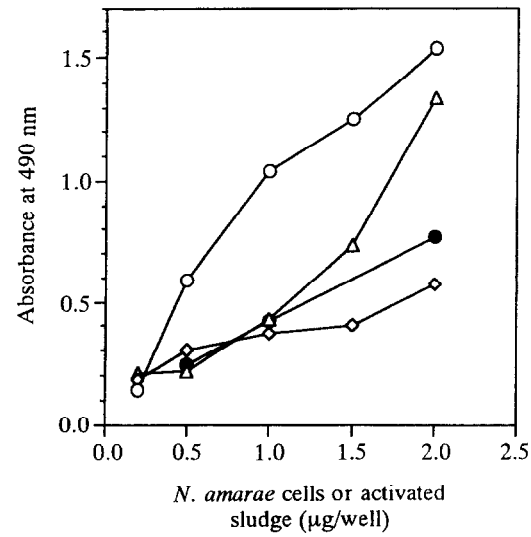

FIG. 2. Antibody titers against *N. amarae* cells (○, ◇, △) and activated sludge (●). Antisera were harvested from rabbits 1 (○ ●), 2 (◇), and 3 (△).

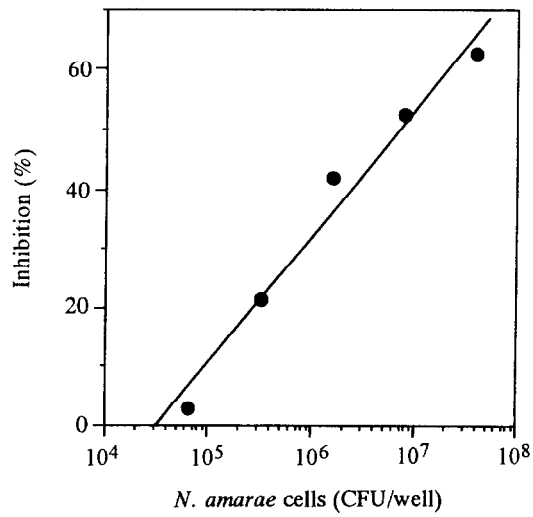

FIG. 3. Detection of *N. amarae* cells by competitive ELISA.

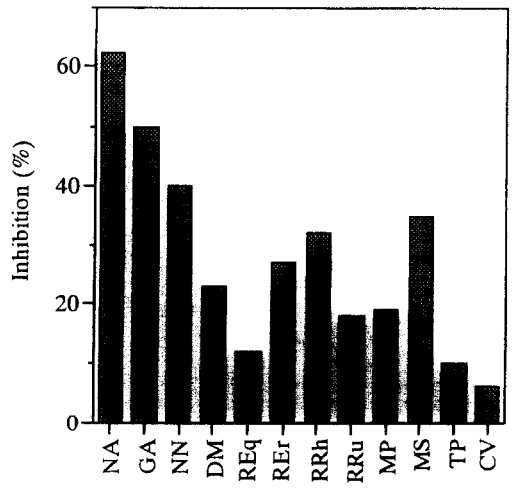

FIG. 4. Cross-reactivity of anti-*N. amarae* antibody against mycolic acid-containing bacteria. For strain abbreviations see Table 1.

anti-*N. amarae* antibody should be widely applicable.

Determination of *N. amarae* in the activated sludge process *N. amarae* cell numbers (CFUs) in samples obtained from the activated sludge process were determined by the plate count method (Table 2). The activated sludge scum contained the largest number of *N. amarae* cells (1.0×10^7 CFU/mg-MLSS), while the secondary effluent content was relatively large (1.3×10^6 CFU/mg-MLSS). These results confirm that *N. amarae* is accumulated in the sludge scum, as previously described (10, 20). Different degrees of inhibition were obtained when the samples were subjected to competitive ELISA. The numbers of *N. amarae* cells were estimated from the inhibition efficiencies using the calibration curve shown in Fig. 3. The results estimated by competitive ELISA corresponded well with those obtained by the plate count method (Fig. 5). The use of anti-*N. amarae* antibody was thus clearly shown to be applicable to estimating cell numbers of *N. amarae* as the dominant species among foam-causing bacteria in the activated sludge process.

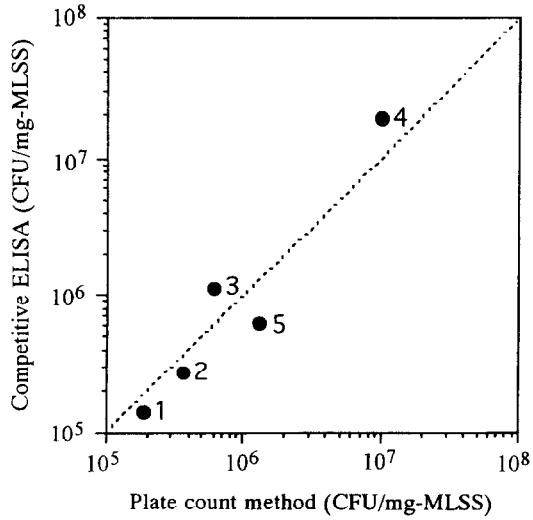

FIG. 5. Estimation of *N. amarae* cells in the activated sludge process by competitive ELISA. The values determined by competitive ELISA are compared with those determined by the plate count method (Table 2). The numbers next to the plots correspond to the sample numbers in Fig. 1.

TABLE 2. *N. amarae* cell numbers in the activated sludge process

Sample	MLSS (mg/*l*)	Water content (%)	*N. amarae* cell number (CFU/ml)	*N. amarae* cell number (CFU/mg-MLSS)
Raw sewage	246	—	4.7×10^4	1.9×10^5
Primary effluent	74	—	2.7×10^4	3.6×10^5
Aeration basin effluent	2090	—	1.3×10^5	6.2×10^5
Scum in secondary clarifier	—	95	—	1.0×10^7
Secondary effluent	7.0	—	9.3×10^3	1.3×10^6

Conclusions In this paper, the production of anti-*N. amarae* antibody and its application to determining *N. amarae* cells in the activated sludge process were investigated. The antibody from rabbit 1 recognized only a small portion of the activated sludge, indicating the possibility of using the antibody for the specific detection of *N. amarae* cells in activated sludge, although its cross-reactivity against gram-negative and gram-positive bacteria other than mycolic acid-containing bacteria was not tested in this study. The applicability of the antibody was actually demonstrated by experiments in which *N. amarae* cells in the activated sludge process were estimated by competitive ELISA. Some countermeasures, including the addition of chemicals and physical procedures, have been adopted to prevent the growth of *N. amarae* in activated sludge. However, to control *N. amarae* growth effectively, its rapid detection and estimation are needed. In conclusion, the ELISA technique developed in this study is seen as a novel and effective method for monitoring *N. amarae* cells in the activated sludge process.

ACKNOWLEDGMENT

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, No. 08650650 (1996). The authors would like to thank the sewage treatment plant for providing samples.

REFERENCES

1. **Soddell, J. A. and Seviour, R. J.:** Microbiology of foaming in activated sludge plants. J. Appl. Bacteriol., **69**, 145–176 (1990).
2. **Stratton, H. M., Seviour, R. J., Soddell, J. A., Blackall, L. L., and Muir, D.:** The opportunistic pathogen *Nocardia farcinica* is a foam-producing bacterium in activated sludge plants. Lett. Appl. Microbiol., **22**, 342–346 (1996).
3. **Lechevalier, M. P. and Lechevalier, H. A.:** *Nocardia amarae* sp. nov., an actinomycete common in foaming activated sludge. Int. J. Syst. Bacteriol., **24**, 278–288 (1974).
4. **Klatte, S., Rainey, F. A., and Kroppenstedt, R. M.:** Transfer of *Rhodococcus aichiensis* Tsukamura 1982 and *Nocardia amarae* Lechevalier and Lechevalier 1974 to genus *Gordona* as *Gordona aichiensis* comb. nov. and *Gordona amarae* comb. nov. Int. J. Syst. Bacteriol., **44**, 769–773 (1994).
5. **Goodfellow, M., Chun, J., Stubbs, S., and Tobili, A. S.:** Transfer of *Nocardia amarae* Lechevalier and Lechevalier 1974 to the genus *Gordona* as *Gordona amarae* comb. nov. Lett. Appl. Microbiol., **19**, 401–405 (1994).
6. **Lemmer, H. and Kroppenstedt, R. M.:** Chemotaxonomy and physiology of some actinomycetes isolated from scumming activated sludge. Syst. Appl. Microbiol., **5**, 124–135 (1984).
7. **Sezgin, M., Lechevalier, M. P., and Karr, P. R.:** Isolation and identification of actinomycetes present in activated sludge

scum. Water Sci. Technol., **20**, 257–263 (1988).
8. **Lemmer, H. and Braumann, M.:** Scum actinomycetes in sewage treatment plants. II. The effect of hydrophobic substrate. Water Res., **22**, 761–763 (1988).
9. **Akit, J., Cooper, D. G., Manninen, K. I., and Zajic, J. E.:** Investigation of potential biosurfactant production among phytopathogenic corynebacteria and related soil microbes. Curr. Microbiol., **6**, 145–150 (1981).
10. **Fujita, M., Iwahori, K., and Taki, H.:** A novel method of enumerating *Nocardia amarae* in foaming activated sludge. J. Ferment. Bioeng., **77**, 674–678 (1994).
11. **Safferman, R. S.:** Standard methods for examination of water and wastewater, 16th ed., p. 933. American Public Health Assoc., Washington, D.C. (1990).
12. **de los Reyes, F. L., Ritter, W., and Raskin, L.:** Group-specific small-subunit rRNA hybridization probes to characterize filamentous foaming in activated sludge systems. Appl. Environ. Microbiol., **63**, 1107–1117 (1997).
13. **de los Reyes, M. F., de los Reyes III, F. L., Hernandez, M., and Raskin, L.:** Quantification of *Gordona amarae* strains in foaming activated sludge and anaerobic digester systems with oligonucleotide hybridization probes. Appl. Environ. Microbiol., **64**, 2503–2512 (1998).
14. **Hernandez, M., Jenkins, D., and Beaman, B. L.:** Mass and viability estimations of *Nocardia* in activated sludge and anaerobic digesters using conventional strains and immunofluorescent methods. Water Sci. Technol., **29**, 249–259 (1994).
15. **Sakai, Y., Mori, T., Iida, M., Honda, K., and Matsumoto, T.:** Scum formation by actinomycetes (*Nocardia* sp.) in final clarifier of activated sludge process. J. Jpn. Sew. Works Assoc., **19**, 56–65 (1982). (in Japanese)
16. **Institute For Fermentation, Osaka:** List of cultures. Microorganisms, 10th ed., p. 498–516. IFO, Osaka (1996).
17. **Orhon, D. and Artan, N.:** Modelling of activated sludge systems, p. 16–18. Technomic Publishing Company, Lancaster, PA (1994).
18. **Japan Sewage Works Association:** Standard methods for the examination of sewage. JSWA, Tokyo (1984). (in Japanese)
19. **Takai, T., Niioka, H., Gao, Y., Matsumura, M., Inamori, Y., and Hirata, A.:** Development of rapid detection and quantification methods for nitrifying bacteria using monoclonal antibodies. J. Jpn. Soc. Water Environ., **20**, 318–323 (1997). (in Japanese)
20. **Iwahori, K., Taki, H., Miyata, N., and Fujita, M.:** Analysis of *Nocardia amarae* profiles in actual foaming activated sludge plant with viable cell count measurement. J. Ferment. Bioeng., **84**, 98–102 (1997).
21. **Soddell, J. A., Knight, G., Strachan, W., and Seviour, R.:** Nocardioforms, not *Nocardia* foams. Water Sci. Technol., **26**, 455–460 (1992).
22. **Blackall, L. L., Palet, J. H., Hayward, A. C., Minnikin, D. E., Greenfield, P. F., and Harbers, A. E.:** *Nocardia pinesis* sp. nov., an actinomycete found in activated sludge foams in Australia. J. Gen. Microbiol., **135**, 1547–1558 (1989).
23. **Dhaliwal, B. S.:** *Nocardia amarae* and activated sludge foaming. J. Water Pollut. Control Fed., **51**, 344–350 (1979).