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A Novel Nicotine-Degrading Bacterium *Pseudomonas fluorescens* Strain 1206[1]

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Abstract—A highly effective nicotine-degrading bacterial strain 1206 was isolated and identified as *Pseudomonas fluorescens* according to its morphological, physiological and biochemical characteristics and 16S rDNA sequence analysis. Strain *P. fluorescens* 1206 could utilize nicotine as the sole carbon and nitrogen source and degrade nicotine while growing in Luria-Bertani medium. This bacterium was able to remove about 33.8, 82.5 and 97.1% of nicotine with initial concentration of 1 g/L after 8, 16 and 24 h of incubation, respectively. This is the first report on the isolation and characterization of a strain of *P. fluorescens* with high-degrading nicotine ability. This finding may open the way to new biotechnological application of *P. fluorescens* to degradation of nicotine.

Keywords: nicotine biodegradation, *Pseudomonas fluorescens*

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Nicotine 3-(1-methyl-2-pyrrolidinyl)pyridine as the primary alkaloid in tobacco plant, constitutes approximately 0.17–4.93% of dry weight of tobacco [1]. China is the largest tobacco producer and consumer in the world (approximately 30–35%). Tobacco use is primarily due to the stimulant effect of nicotine, and proper concentration of nicotine is responsible for the smoking properties. On the other hand, nicotine can pollute environment and cause serious ecological problems. Nicotine and its derivatives are known to be toxic to many living organisms. The tobacco manufacturing process and all activities that use tobacco often produce a lot of solid or liquid wastes containing high concentration of nicotine. Its main characteristics are the high nicotine content as the only toxic compound [2]. It is very important for development of tobacco manufacture and environment protection how to remove/adjust nicotine. Several methods including natural degradation, chemical-physical processes [3, 4], and biological degradation have been developed for nicotine removal and/or detoxification. The biological degradation of nicotine using microorganisms has been attracted growing attention due to its high efficiency and simple processing. There have been numerous studies on nicotine degradation by microbial treatment in the past.

[1] The article is published in the original.

Many microorganisms have been found to be able to degrade nicotine, e.g., *Arthrobacter nicotinoborans* [5], *A. globiformils* [6], *Nocardioides* sp. [7], *Achromobacter nicotinophagum* [8], *Rhodococcus* sp. [9], *Ochrobactrum intermedium* [10], *Ensifer* sp. [11]. Moreover, a wide range of *Pseudomonas* species and strains have shown capable of degrading nicotine [12], for example, *Pseudomonas putida* [13, 14], *Pseudomonas convexa* [15], *Pseudomonas* sp. Nic22 [16], and *Pseudomonas* sp. HF-1 [17, 18]. To our knowledge, there is no report on nicotine degradation by *Pseudomonas fluorescens*. In this paper, a novel nicotine-degrading bacterium, *P. fluorescens* strain 1206, was isolated and identified, and conditions for degradation of pure nicotine were characterized.

MATERIALS AND METHODS

Chemicals and media. (s)-Nicotine (>99.0% pure) was bought from Sigma-Aldrich (USA). All other chemical reagents were of analytical grade. Taq enzymes, miniBEST bacterial genomic DNA extraction kit, and DNA A-tailing kit were purchased from TaKaRa Biotechnology Co., Ltd. (China). The water used was double deionized water.

The enrichment medium (EM) contained (g/L): K_2HPO_4—1.6, KH_2PO_4—0.4, NaCl—0.1, $MgSO_4 \cdot 7H_2O$—0.2, and $CaCl_2$—0.05. The pH was adjusted to

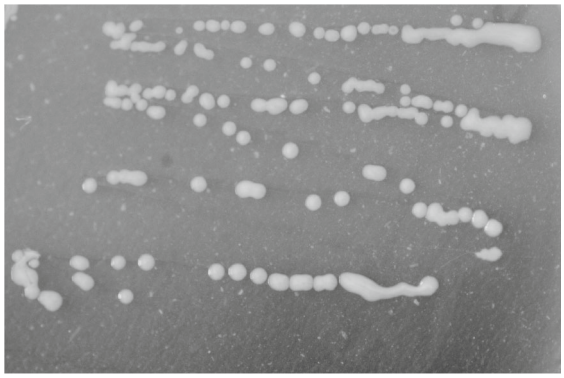

Fig. 1. The colony morphology of the strain 1206.

7.0 and, after autoclaving, the medium was supplemented with 1 mL filter-sterilized nicotine.

The isolation medium (IM) consisted of one liter of EM supplied with 1 mL trace elements solution containing (g/L): $MnSO_4 \cdot H_2O$—2.0, $CuSO_4 \cdot 5H_2O$—0.1, $ZnSO_4 \cdot 7H_2O$—0.2, $NaMoO_4 \cdot 2H_2O$—0.2 and agar—15.0.

The Luria-Bertani (LB) medium was composed of (g/L): tryptone—10.0, yeast extract—5.0 and NaCl—5.0.

The Luria-Bertani-nicotine (LB-N) medium contained one liter of LB supplied with 1 mL nicotine.

Enrichment and bacterial isolation. Soil samples were collected from tobacco fields (Yunnan, China). Bacterial strains were isolated by the following selective enrichment procedures. The sample was incubated in EM medium on the shaker at 30°C and 200 rpm for 3 days. The spread-plate method was used for isolation of pure strains using IM. The homogenous colonies were obtained by taking and then transferring single colonies into new plates.

Morphological and biochemical tests. Clone morphology of the bacterial strain 1206 was determined after 36 h incubation on NA medium. Cell morphology was observed by transmission electron microscope JEM100CX-II (JEOL Ltd. Japan) after staining negatively with 2% (wt/vol) ammonium molybdate. Conventional physiological and biochemical characteristic assays were carried out according to the procedures described in the Bergey's Manual of Determinative Bacteriology [19].

Phylogenetic analysis. Bacterial DNA was extracted from an overnight culture using TaKaRa miniBEST bacterial genomic DNA extraction kit (Ver.2.0), according to the manufacturer's instructions. 16S rDNA gene of the strain 1206 was amplified using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') [20]. The PCR products were purified from the agarose gel using gel extraction kit (TaKaRa, China) and then cloned employing a pMD™18-T vector cloning kit (TaKaRa, China) in accordance with the manufacturer's instructions. Plasmid DNAs containing inserts were sequenced by TaKaRa Biotechnology Co., Ltd. (China).

Related 16S rDNA sequences were compared and obtained from the GenBank database [21] using the BLAST search program [22]. 16S rDNA sequences were initially aligned using multiple sequence alignment software CLUSTAL-W [23]. The phylogenetic tree was constructed using MEGA 3.0 software [24] based on the 16S rDNA sequences of closely related strains to the strain 1206, and *Xanthomonas retroflexus* was as the out group.

Growth and nicotine degradation. The strain 1206 was incubated in LB or LB-N medium with 3 independent replicates at 25°C and 150 rpm. The reported values represent the average of 3 different readings of each experiment. The initial bacteria concentration was about 1×10^8 CFU/mL. Samples were taken every 2 h to determine the growth rate and the concentration of nicotine.

Cell growth was monitored spectrophotometrically by measuring the OD_{600}.

Nicotine analysis was performed using a high-pressure liquid chromatography (HPLC) (Agilent 110 Series, USA), equipped with an SB C18 column (5 μm 4.6 × 150 mm) and a DAD detector at 260 nm wavelength. Sample was eluted with a linear gradient from 90 to 20% for solvent A (50 mM KH_2PO_4) and from 10 to 80% for solvent B (CH_3OH) over 15 min at a flow rate of 0.8 mL/min.

RESULTS AND DISCUSSION

Cellular and colony morphology of the strain 1206. Nicotine-degrading bacteria were isolated using IM medium where the nicotine was the sole carbon and nitrogen source for growth. The bacterial isolates showed abundant diversity of nicotine-degrading bacterial community according to colonies morphology (data not shown). A homogeneous, white-yellow, round with a glassy surface and smooth edges, opaque colonies, each of which was approximately 2.00 mm in diameter (Fig. 1), were observed on the nutrient agar medium plates after 36 h inoculation. The strain was named 1206 and selected for further identification and characterization.

The cells of strain 1206 were non-spore-forming, approximately 0.8–1.0 × 1.8–2.5 μm in size, with multiple polar flagella (Fig. 2), rod-shaped bacteria similar to the member of genus *Pseudomonas*.

Identification of the bacterial strain 1206. The 16S rRNA gene of strain 1206 was sequenced and deposited into GenBank under the accession number GU059580. A phylogenetic tree was constructed based on the 16S rDNA sequences of related bacterial species (Fig. 3). 16S rRNA gene of 1206 analysis revealed members of the genus *Pseudomonas* as the

closest relatives with more than 99% identity, especially closely related to *P. putida*, *P. koreensis*, *P. clemancea, P. jessenii, P. pavonaceae* and *P. fluorescens*. The relationships between these species were so close that it was difficult to determine which species the strain 1206 belonged to. Although it is not sufficient to place the strain 1206 to the species level, it does match the isolated strain to the *Pseudomonas* genus.

The strain 1206 was a Gram-negative, oxidase positive, aerobic bacterium, able to reduce nitrate to nitrite. Strain 1206 could utilize glucose, sucrose, trehalose, maltose, mannitol and inositol. The gelatin liquefaction test was negative. Other results of morphological and physiological characterization tests of the strain 1206 are summarized in Table 1. The results were identical to those of *P. fluorescens* according to *Bergey's Manual of Determinative Bacteriology* [19].

Based on the biochemical and morphological characteristics, and the analysis of 16S rRNA gene sequence, strain 1206 was identified as *P. fluorescens*, which was deposited in the China General Microbiological Culture Collection Center (No. CGMCC 3149).

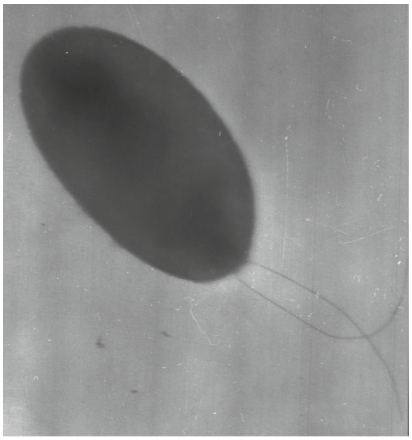

Fig. 2. Transmission electron micrographs of negatively stained cells of the strain 1206.

To date, a number of different *Pseudomonas* species that are capable of degrading nicotine, such as *P. putida* [13, 14], *P. convexa* [15], *Pseudomonas* sp. Nic22 [16], and *Pseudomonas* sp. HF-1 [17, 18] have

Table 1. Physiological and biochemical characteristics of strain 1206

Test characters	*P. fluorescens*	*P. clemancea*	*P. putida*	The strain 1206
Fluorescent, diffusible pigment	+	–	+	+
Diffusible non-fluorescent pigment	–	n.d.	–	–
Gram staining	–	n.d.	–	–
Growth at 41°C	–	n.d.	–	–
Grows aerobically	+	+	+	+
Growth at 4°C	+	+	+	+
Arginine dihydrolase	+	n.d.	+	+
Oxidase reaction	+	+	+	+
Gelatine liquefication	+	–	–	+
Starch hydrolysis	–	–	–	–
Urease	–	n.d.	–	–
Levan formation from sucrose	n.d.	n.d.	n.d.	+
Nitrate reduction	+	+	–	+
Utilization of				
Glucose	+	+	+	+
Sucrose	+	n.d.	–	+
Trehalose	+	n.d.	–	+
Maltose	+	n.d.	–	+
Mannitol	+	n.d.	–	+
Inositol	+	n.d.	–	+
L-Valine	+	n.d.	+	+
L-Proline	+	n.d.	+	+
β-Alanine	+	n.d.	+	+
L-Arginine	+	n.d.	n.d.	+

n.d.—not done.

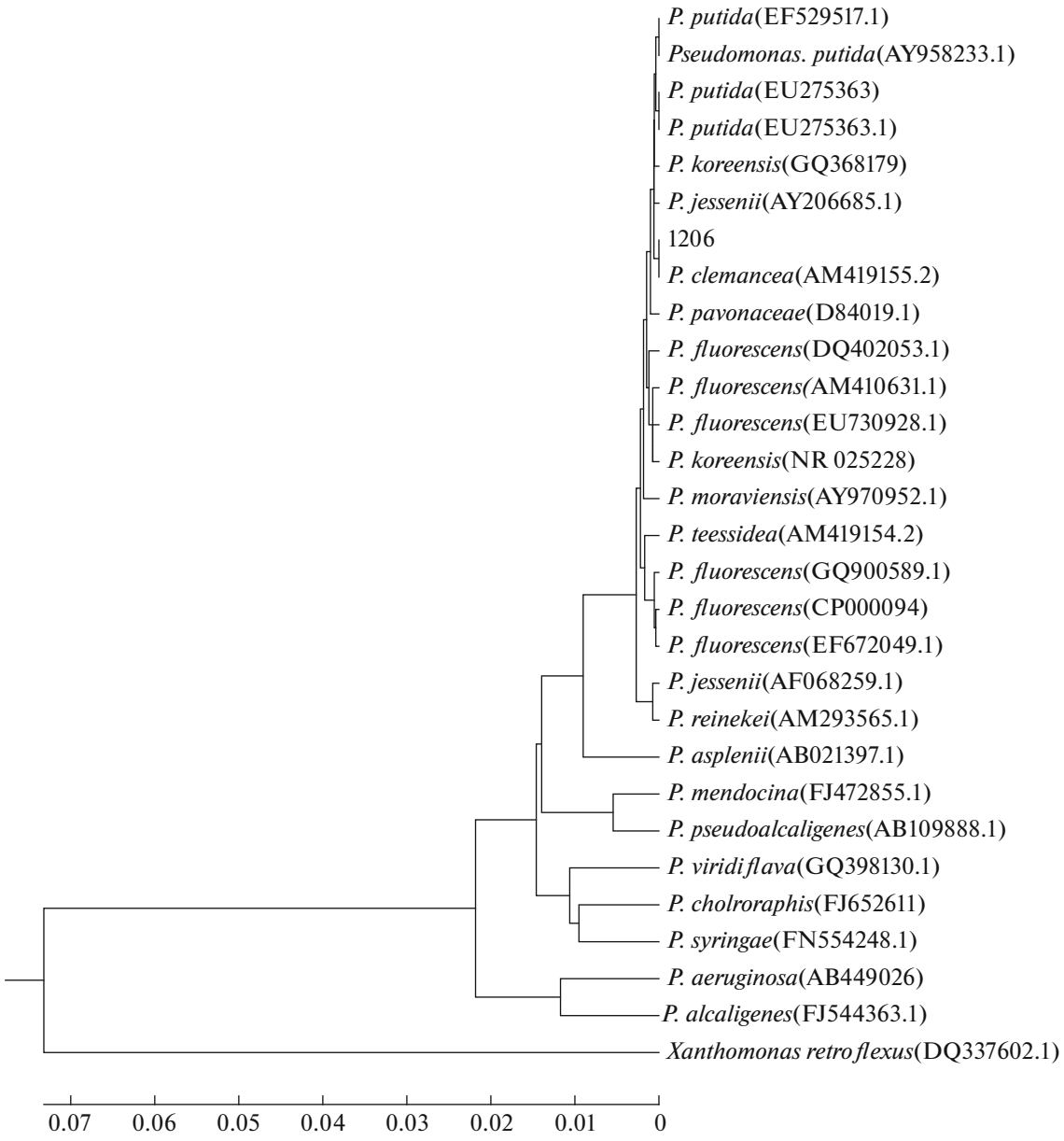

Fig. 3. Phylogenetic tree derived from the 16S rDNA sequences of *Pseudomonas* species related to the strain 1206. The *X. retroflexus* DQ337602 as an out group. The accession numbers of the strains were showed after the species name.

been reported. However, the isolation of *P. fluorescens* capable of nicotine degradation had not been reported before our study. *P. fluorescens* is a versatile bacterium that grows in soil, coastal marine habitats, and on plant and animal tissues. Several *P. fluorescens* strains (e.g., CHA0 or Pf-5) demonstrate biocontrol properties, protecting the roots of some plant species against parasitic fungi such as *Fusarium* or *Pythium*, as well as some plant-parasitic nematodes [25]. Production of secondary metabolites plays an important role in plant disease suppression. Antibiotics such as pyrrolnitrin, pyoluteorin, and 2,4-diacetylphloroglucinol that inhibit phytopathogen growth are produced by *P. fluorescens* [26]. The *P. fluorescens* has been applied to degradation of pollutants such as styrene [27], 2,4,6-trinitrotoluene [28] and polycyclic aromatic hydrocarbons [29].

Growth and nicotine-degrading property of the strain 1206. As shown in Fig. 4, more than 97% of the nicotine was degraded by *P. fluorescens* 1206 after 24 h incubation on LB-N medium at 25°C. A good correlation was observed between the increase of the OD_{600} value of the bacterial culture and the decrease of the nicotine concentration in LB-N medium. In the initial 8 h, nicotine was degraded slowly but the degrada-

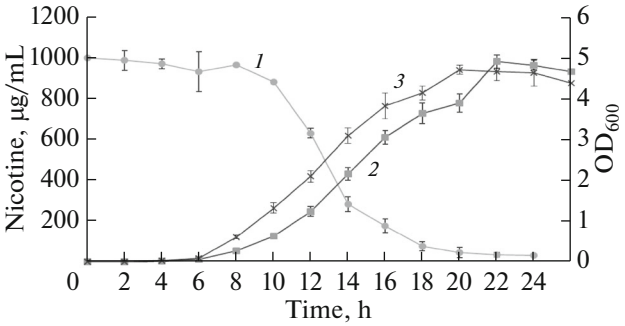

Fig. 4. Growth and nicotine degradation curves of *P. fluorescens* 1206. *1*—Nicotine concentration degradation by 1206; *2*—cell growth of 1206 in LB-N medium; *3*—cell growth of 1206 in LB medium. The values are means of 3 replicates, and the error bars indicate the standard deviations.

tion rate was accelerated in the period from 8 to 18 h. The *P. fluorescens* 1206 showed diverse growth patterns in different media during incubation. These bacteria grew better in LB medium than in LB-N medium, which indicated that nicotine was likely to be toxic to strain 1206 and inhibited the growth of the strain slightly. After 12 h of incubation on nutrient agar medium, the growth rate of strain 1206 increased gradually along with the degradation of nicotine.

In this study, Gram-negative bacterial strain 1206 which can use nicotine as sole carbon and energy sources was isolated from the soil collected from tobacco fields (Yunnan,China). It was identified as *P. fluorescens* by morphological, biochemical and 16S rDNA sequence analysis. This study firstly reports the isolation and characterization of *P. fluorescens* 1206 capable of degrading nicotine. *P. fluorescens* 1206 was able to remove about 33.8, 82.5 and 97.1% of nicotine with initial concentration of 1 g/L after 8, 16 and 24 h of incubation, respectively. It was also noted that the presence of nicotine in LB medium had inhibitory effect on bacterial growth. The further research will be focus on elucidating the molecular mechanisms of nicotine degradation by *P. fluorescens* 1206. This will allow the subsequent development of molecular tools to screen both natural environmental and engineered processes for tobacco waste treatment.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

REFERENCES

1. Tso, T.C., *Tob New York*, 1969, no. 4, pp. 69–73.
2. Civilini, M., Domenis, C., Sebastianutto, N., and Bertoldi, M., *Waste Manage. Res.*, 1997, vol. 15, no. 4, pp. 349–358.
3. US Patent no. 3139435, 1964.
4. US Patent no. 4848373, 1989.
5. Decker, K. and Bleeg, H., *Biochem. Biophys. Acta,* 1965, vol. 105, no. 2, pp. 313–324.
6. Uchida, S., Maeda, S., Masubuchi, K., Shinohara, T., and Kisaki, T., *J. Sci. Pap.*, 1976, vol. 118, pp. 197–201.
7. Ganas, P., Sachelaru, P., Mihasan, M., Igloi, G.L., and Brandsch, R., *Arch. Microbiol.*, 2008, vol. 189, no. 5, pp. 511–517.
8. Hylin, J. W., *Arch. Biochem. Biophys.*, 1959, vol. 83, no. 2, pp. 528–537.
9. Gong, X.W., Yang, J.K., Duan, Y.Q., Dong, J.Y., Zhe, W., Wang, L., et al., *Res. Microbiol.*, 2009, vol. 160, no. 3, pp. 200–204.
10. Yuan, Y.J., Lu, Z.X., Wu, N., Huang, L.J., Lu, F.X., and Bie, X.M., *Int. Biodeter. Biodegr.*, 2005, vol. 56, no. 1, pp. 45–50.
11. Lei, L.P., Zhang, W., Wei, H.L., Xia, Z.Y., and Liu, X.Z., *Ann. Microbiol.,* 2009, vol. 59, no. 2, pp. 247–252.
12. Li, H.J., Li, X.M., Duan, Y.Q., Zhang, K.Q., and Yang, J.K. *Appl. Microbiol. Biotechnol.*, 2010, vol. 86, no. 1, pp. 11–17.
13. Wang, S.N., Liu, Z., Tang, H.Z., Meng, J., and Xu, P., *Microbiology*, 2007, vol. 153, no. 5, pp. 1556–1565.
14. Wei, H.L., Lei, L.P., Xia, Z.Y., Liu, S., Liu, P.G., and Liu, X.Z., *Ann. Microbiol.*, 2008, vol. 58, no. 1, pp. 41–45.
15. Thacker, R., Rørvig, O., Kahlon, P., and Gunsalus, I.C., *J. Bacteriol.*, 1978, vol. 135, no. 1, pp. 289–290.
16. Chen, C.M., Li, X.M., Yang, J.K., Gong, X.W., Li, B., and Zhang, K.Q. *Int. Biodeter. Biodegr.*, 2008, vol. 62, no. 3, pp. 226–231.
17. Ruan, A.D., Min, H., Peng, X.H., and Huang, Z., *Res. Microbiol.*, 2005, vol. 156, no. 5, pp. 700–706.
18. Wang, M.Z., Yang, G.Q., Min, H., and Lv, Z.M., *Can. J. Microbiol.*, 2009, vol. 55, no. 3, pp. 228–233.
19. Bergey D.H. and Holt J.G. *Bergey's Manual of Determinative Bacteriology,* Baltimore: Williams and Wilkins, 1994.

20. *Nucleic Acid Techniques in Bacterial Systematics*, Stackebrandt, E. and Goodfellow, M., Eds., New York: John Wiley and Sons, 1991.
21. Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., Rapp, B.A., and Wheeler, D.L., *Nucleic Acids Res.*, 2002, vol. 30, no. 1, pp. 17–20.
22. Altschul, S.F., Madden, T.L., Schaffer, A.A, Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. *Nucleic Acids Res.*, 1997, vol. 25, no. 17, pp. 3389–3402.
23. Thompson, J.D., Higgins, D.G., and Gibson, T.J., *Nucleic Acid Res.*, 1994, vol. 22, no. 22, pp. 4673–4680.
24. Kumar, S., Tamura, K., and Nei, M., *Briefings Bioinf.*, 2004, vol. 5, no. 2, pp. 150–163.
25. Haas, D. and Keel, C., *Annu. Rev. Phytopathol.*, 2003, vol. 41, no. 1, pp. 117–153.
26. Paulsen, I.T., Press, C.M., Ravel, J., Kobayashi, D.Y., Myers, G.S.A., Mavrodi, D.V., et al., *Nat. Biotechnol.*, 2005, vol. 23, no. 7, pp. 873–878.
27. Di Gennaro, P., Ferrara, S., Ronco, I., Galli, E., Sello, G., Papacchini, M., and Bestetti, G., *Arch. Microbiol.*, 2007, vol. 188, no. 2, pp. 117–125.
28. Naumova, R.P., Selivanovskaia, S., and Mingatina, F.A., *Mikrobiologiia*, 1988, vol. 57, no. 2, pp. 218–222.
29. Bugg, T., Foght, J.M., Pickard, M.A., and Gray, M.R., *Appl. Environ. Microbiol.*, 2000, vol. 66, no. 12, pp. 5387–5392.