Applied and Environmental Microbiology, Nov. 2009, p. 6896–6899 Vol. 75, No. 21
0099-2240/09/$12.00 doi:10.1128/AEM.00450-09

Biogenic Formation of As-S Nanotubes by Diverse *Shewanella* Strains▽†

Shenghua Jiang,[1]‡ Ji-Hoon Lee,[1]‡§ Min-Gyu Kim,[3] Nosang V. Myung,[4] James K. Fredrickson,[5] Michael J. Sadowsky,[6] and Hor-Gil Hur[1,2]*

Department of Environmental Science and Engineering[1] and International Environmental Research Center,[2] Gwangju Institute of Science and Technology, Gwangju 500-712, Republic of Korea; Pohang Accelerator Laboratory, Pohang, Gyeongbuk 790-784, Republic of Korea[3]; Department of Chemical and Environmental Engineering and Center for Nanoscale Science and Engineering, University of California at Riverside, Riverside, California 92521[4]; Biological Sciences Division, Pacific Northwest National Laboratory, Richland, Washington 99352[5]; and Department of Soil, Water, and Climate and BioTechnology Institute, University of Minnesota, St. Paul, Minnesota 55108[6]

Received 23 February 2009/Accepted 22 August 2009

***Shewanella* sp. strain HN-41 was previously shown to produce novel, photoactive, As-S nanotubes via the reduction of As(V) and $S_2O_3^{2-}$ under anaerobic conditions. To determine if this ability was unique to this bacterium, 10 different *Shewanella* strains, including *Shewanella* sp. strain HN-41, *Shewanella* sp. strain PV-4, *Shewanella alga* BrY, *Shewanella amazonensis* SB2B, *Shewanella denitrificans* OS217, *Shewanella oneidensis* MR-1, *Shewanella putrefaciens* CN-32, *S. putrefaciens* IR-1, *S. putrefaciens* SP200, and *S. putrefaciens* W3-6-1, were examined for production of As-S nanotubes under standardized conditions. Of the 10 strains examined, three formed As-S nanotubes like those of strain HN-41. While *Shewanella* sp. strain HN-41 and *S. putrefaciens* CN-32 rapidly formed As-S precipitates in 7 days, strains *S. alga* BrY and *S. oneidensis* MR-1 reduced As(V) at a much lower rate and formed yellow As-S after 30 days. Electron microscopy, energy-dispersive X-ray spectroscopy, and extended X-ray absorption fine-structure spectroscopy analyses showed that the morphological and chemical properties of As-S formed by strains *S. putrefaciens* CN-32, *S. alga* BrY, and *S. oneidensis* MR-1 were similar to those previously determined for *Shewanella* sp. strain HN-41 As-S nanotubes. These studies indicated that the formation of As-S nanotubes is widespread among *Shewanella* strains and is closely related to bacterial growth and the reduction rate of As(V) and thiosulfate.**

A number of bacterial strains have been shown to contribute to the formation of diverse arsenic minerals (4). If sulfide is present as a ligand for immobilization of arsenic, As-S precipitates often form. *Desulfosporosinus auripigmentum*, which can be isolated from lake sediments, reduces As(V) to As(III) and S(VI) to S(−II) during anaerobic respiration and forms a yellow arsenic sulfide precipitate (7). While *Desulfovibrio* strain Ben-RB also produces precipitated arsenic sulfide in culture media, As reduction was not correlated with energy conservation (6). Other taxonomically divergent microorganisms isolated from various arsenic-rich sites have also been shown to reduce As(V) to As(III) and form arsenic sulfide precipitates (1, 2).

We previously reported that *Shewanella* sp. strain HN-41 produces an extensive extracellular network of filamentous arsenic-sulfide (As-S) nanotubes via its dissimilatory metal-reducing activity (4). The As-S nanotubes, which formed via the reduction of As(V) and $S_2O_3^{2-}$, were initially amorphous As_2S_3 but evolved with increasing incubation time toward polycrystalline phases of the chalcogenide minerals realgar (AsS) and duranusite (As_4S). Because the *Shewanella* As-S nanotubes behaved both as metals and as semiconductors, in terms of their electrical and photoconductive properties, respectively, it was postulated that they may provide useful materials for novel nano- and optoelectronic devices (4).

While several bacterial species have been shown to produce amorphous and particulate As-S precipitates (1, 2, 4, 7), the formation of the As-S nanotubes by other bacteria has not yet been described, suggesting that this may be a unique property of *Shewanella* strains. To test this hypothesis, 10 different *Shewanella* strains, including *Shewanella* sp. strains PV-4 and HN-41, *Shewanella alga* BrY, *Shewanella amazonensis* SB2B, *Shewanella denitrificans* OS217, *Shewanella oneidensis* MR-1, *Shewanella putrefaciens* CN-32, *S. putrefaciens* IR-1, *S. putrefaciens* SP200, and *S. putrefaciens* W3-6-1, were inoculated into HEPES-buffered basal medium (3, 5) containing 10 mM sodium DL-lactate as the electron donor and 5 mM arsenate ($Na_2HAsO_4 \cdot 7H_2O$) and 5 mM thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) as the electron acceptors. All chemicals and methods for sample preparation and characterization used in this study were previously described (4).

Of the 10 different *Shewanella* strains examined, only four strains, *Shewanella* sp. strain HN-41, *S. putrefaciens* CN-32, *S. alga* BrY, and *S. oneidensis* MR-1, produced As-S yellow precipitates in culture medium following incubation in the presence of arsenate and thiosulfate. *Shewanella* sp. strain HN-41 and *S. putrefaciens* CN-32 produced yellow precipitates of As-S after 7 days of incubation, whereas *S. alga* BrY and *S. onei-*

* Corresponding author. Mailing address: Department of Environmental Science and Engineering, Gwangju Institute of Science and Technology, Gwangju 500-712, Republic of Korea. Phone: 82-62-970-2437. Fax: 82-62-970-2434. E-mail: hghur@gist.ac.kr.
‡ S.J. and J.-H.L. contributed equally to this work.
§ Present address: Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA 99352.
† Supplemental material for this article may be found at http://aem.asm.org/.
▽ Published ahead of print on 28 August 2009.

TABLE 1. Influence of thiosulfate on the consumption of lactate, reduction of As(V), and formation of As-S nanotubes by *Shewanella* strains in medium containing lactate and 5 mM As(V)

Shewanella strain	Consumption of lactate in medium supplemented with:		Reduction of As(V) in medium supplemented with:		Formation of As-S nanotubes in medium supplemented with As(V) and $S_2O_3^{2-}$ after:	
	$S_2O_3^{2-}$	No $S_2O_3^{2-}$	$S_2O_3^{2-}$	No $S_2O_3^{2-}$	7 days	30 days
Shewanella sp. strain HN-41	+	−	+	−	+	+
Shewanella sp. strain PV-4	−	−	−	−	−	−
S. alga BrY	+	−	+	−	−	+
S. amazonensis SB2B	−	−	−	−	−	−
S. denitrificans OS217	−	−	−	−	−	−
S. oneidensis MR-1	+	−	+	−	−	+
S. putrefaciens CN-32	+	+	+	+	+	+
S. putrefaciens IR-1	−	−	−	−	−	−
S. putrefaciens SP200	−	−	−	−	−	−
S. putrefaciens W3-6-1	−	−	−	−	−	−

densis MR-1 produced only a small amount of visible precipitate after 30 days of incubation. The remainder of the tested *Shewanella* strains failed to produce yellow precipitates, regardless of incubation time.

The culture medium of the strains tested was periodically sampled during the bacterial incubation period to determine the concentrations of lactate, acetate, arsenic, and sulfide in the aqueous solution. Among the 10 strains examined, *Shewanella* strain HN-41, *S. putrefaciens* CN-32, *S. alga* BrY, and *S. oneidensis* MR-1 metabolized lactate in growth medium containing arsenate and thiosulfate (Table 1). *Shewanella* sp. strain HN-41 and *S. putrefaciens* CN-32 rapidly consumed lactate both as an electron donor and as a carbon source (see Fig. S1 in the supplemental material). Cultures of *S. alga* BrY and *S. oneidensis* MR-1 consumed ~1.4 mM lactate after 7 days, while *Shewanella* sp. strain HN-41 and *S. putrefaciens* CN-32 consumed 1.7 mM and 2.3 mM lactate, respectively. Although *S. putrefaciens* CN-32 reduced As(V) in the culture medium supplemented with 5 mM As(V) as the sole electron acceptor, *Shewanella* sp. strain HN-41, *S. alga* BrY, and *S. oneidensis* MR-1 did not reduce As(V) and did not oxidize lactate to acetate (data not shown). Consequently, the latter three strains could not utilize As(V) as an electron acceptor for respiratory metabolism.

In the presence of thiosulfate, however, *Shewanella* sp. strain HN-41 and *S. putrefaciens* CN-32 reduced As(V) to As(III) and thiosulfate to sulfide, and the lactate consumed was oxidized to acetate. *Shewanella* sp. strain HN-41 and *S. putrefaciens* CN-32 reduced 1.7 and 3 mM As(V) to As(III), respectively, based on determination of As(V) present at day 7. The reduction of As(V) by *S. alga* BrY (0.8 mM) and *S. oneidensis* MR-1 (0.5 mM) was relatively slower than that by *Shewanella* sp. strain HN-41 and *S. putrefaciens* CN-32 (see Fig. S1 in the supplemental material). The sulfide produced in aqueous phase by *Shewanella* sp. strain HN-41 and *S. putrefaciens* CN-32 initially increased to 150 μM and thereafter decreased to 20 μM, concomitantly with the formation of As-S precipitates (see Fig. S2 in the supplemental material).

The As-S nanotubes produced by the *Shewanella* strains were examined for morphology by using scanning electron microscopy and for chemical analysis by using extended X-ray absorption fine-structure (EXAFS) spectroscopy at the Pohang Accelerator Laboratory in Pohang, Republic of Korea (4). Electron microscopic analyses revealed that *S. alga* BrY, *S. oneidensis* MR-1, and *S. putrefaciens* CN-32 produced filamentous As-S nanotubes (Fig. 1), similar to those formed by *Shewanella* sp. strain HN-41 (4). Energy-dispersive X-ray spectral analysis of single, filamentous, As-S nanotubes formed by *S. alga* BrY, *S. oneidensis* MR-1, and *S. putrefaciens* CN-32 showed As/S ratios of 1.23 ± 0.13, 1.34 ± 0.09, and 0.80 ± 0.03, respectively, which were greater than that (0.72 ± 0.03) found in the nanotubes produced by *Shewanella* sp. strain HN-41 (values are means ± standard deviations of six As-S nanotubes from each sample).

The main mineralogical components of the filamentous As-S nanotubes formed by *S. alga* BrY, *S. oneidensis* MR-1, and *S. putrefaciens* CN-32 were comprised of a mixture of several arsenic-rich As-S compounds, with increasing ratios of As to S (see above). The size distribution for the width of the As-S nanotubes formed by *Shewanella* sp. strain HN-41, *S. putrefaciens* CN-32, *S. alga* BrY, and *S. oneidensis* MR-1 was determined by measurement of 100 As-S nanotubes of each sample. Results of this analysis indicated that the As-S nanotubes had a major distribution range of 40 to 70 nm for *Shewanella*. sp. strain HN-41, whereas the other three strains examined produced nanotubes with widths of 30 to 60 nm (Fig. 2).

Radial structure functions of the EXAFS spectra of the As-S nanotubes produced by *S. alga* BrY, *S. oneidensis* MR-1, and *S. putrefaciens* CN-32 showed single crest-peaks corresponding to As(III)-S(−II) bonding, similar to what was seen for the As-S nanotubes produced by *Shewanella*. sp. strain HN-41 (Fig. 3). Additional peaks found in the EXAFS data indicated that there were slight differences among the minerals formed by the strains.

The influence of temperature on the properties and formation of the As-S nanotubes by strains HN-41 and CN-32 was investigated. In addition to forming As-S nanotubes at 20°C, the two strains also formed As-S particle structures (see Fig. S3 in the supplemental material). Moreover, bacterial cultures incubated at 20°C produced about a twofold-greater concentration of sulfide in the liquid medium than that found at 30°C (see Fig. S4 in the supplemental material). Energy-dispersive X-ray spectroscopy analyses showed that the As-S particles produced at 20°C had an As/S ratio similar to that of the As-S

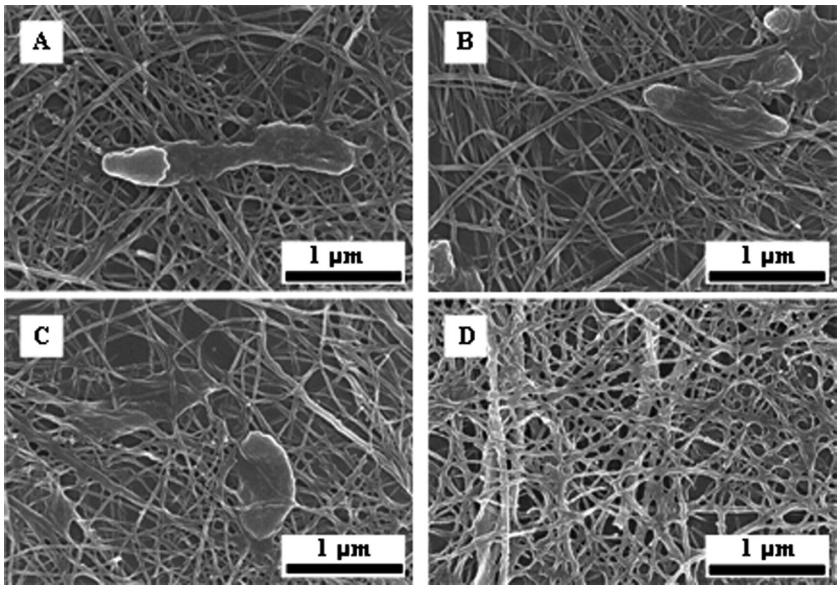

FIG. 1. Scanning electron microscopic images of As-S nanotubes formed by *Shewanella* sp. strain HN-41 (A), *S. putrefaciens* CN-32 (B), *S. alga* BrY (C), and *S. oneidensis* MR-1 (D). Bars, 1 μm.

nanotubes produced at 30°C (data not shown). Mineralogical alteration of the As-S nanotubes with time was also demonstrated by previous X-ray diffraction analyses, in which the ratio of As to S in the precipitates increased with time (4). This resulted in the formation of arsenic-rich phases consisting of As_4S_5, AsS, and As_4S_3. Taken together, these results indicate that physiological properties of the strains and abiological factors, including pH and concentration of S(−II) in the medium, also likely control the varied structures, properties, and stability of the As-S minerals and nanotubes formed by *Shewanella* strains (7).

In the past several years, various As-reducing microorganisms have been isolated (8, 9, 14, 15) and arsenic reduction has been explained by two mechanisms of respiratory and detoxification activities encoded by *arr* and *ars* genes, respectively (13). *Shewanella* sp. strain ANA-3 has been extensively studied to examine mechanisms of arsenate reduction (10–12).

In order to investigate the possible relationship between formation of the As-S nanotubes and arsenate reduction, four different *Shewanella* strains, which appeared to form the As-S nanotubes, were analyzed for the presence and structure of putative *arrA* and *arsC* genes found in the arsenic resistance operon found in *Shewanella* sp. strain ANA-3 (AY271310) (see Table S1 in the supplemental material). The ArrA and ArsC of *Shewanella*. sp. strain HN-41 and *S. putrefaciens* strain CN-32

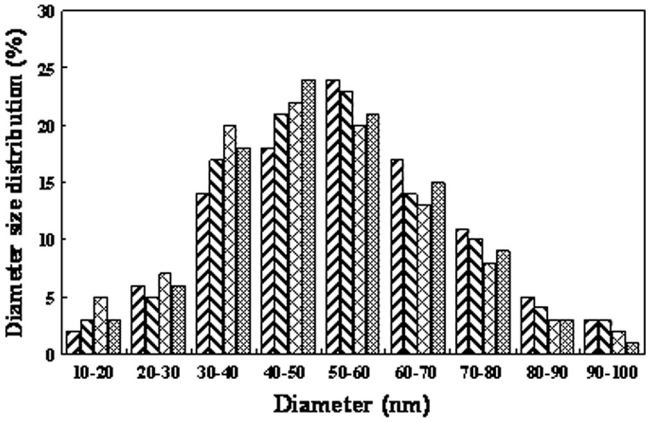

FIG. 2. Diameter size distribution of As-S nanotubes produced by *Shewanella* sp. strain HN-41 (▨), *S. putrefaciens* CN-32 (▧), *S. alga* BrY (▩), and *S. oneidensis* MR-1 (▩). Diameter values were determined from the measurement of 100 As-S nanotubes.

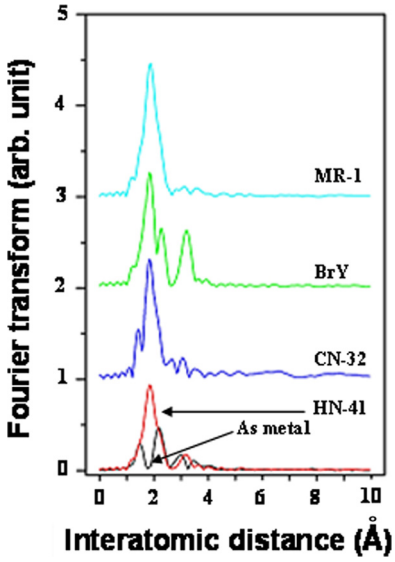

FIG. 3. Fourier-transformed radial structure functions (in *R*-space Å) of EXAFS data from As metal and As-S nanotubes produced by *Shewanella* sp. strain HN-41, *S. putrefaciens* CN-32, *S. alga* BrY, and *S. oneidensis* MR-1.

showed 35.6 and 100%, and 93.7 and 100% protein sequence similarities, respectively, with the corresponding proteins encoded by the *arr-ars* operon from *Shewanella* sp. strain ANA-3 (AY271310). In contrast, *S. oneidensis* MR-1 did not have an identifiable *arrA* gene but contained a putative *arsC* gene with less than 60% protein sequence similarity with the ArsC from *Shewanella* sp. strain ANA-3. The genomic sequence of *S. alga* BrY is not available. While the mechanisms leading to the delayed formation of the As-S nanotubes by *S. oneidensis* MR-1 are not clearly understood, the rapid formation of the As-S nanotubes by *Shewanella* sp. strain HN-41 and *S. putrefaciens* CN-32 may be due to active arsenate reductase systems that are correlated with the presence of the *arrA* and/or *arsC* genes. Since control studies indicated that sulfide alone in a 20 mM concentration was not able to reduce arsenate (data not shown), arsenate reductase activity may be involved in formation of the As-S nanotubes by *Shewanella*. In addition, thiosulfate reduction may also influence the formation of As-S nanotubes.

In summary, the results of the current study indicate that several species and strains of *Shewanella* are able to synthesize As-S nanotubes via the combined reduction of arsenate and thiosulfate. Aside from important biogeological implications, the biogenic formation of one-dimensional As-S nanotubes may also greatly contribute to new, green, biosynthetic methods for the production of inorganic materials at nanoscales, which ultimately may find use in novel nano- and optoelectronic devices. However, to more fully utilize these new materials, more detailed physiological and biochemical studies are needed to better elucidate the mechanisms leading to the biogenic formation of the As-S nanotubes.

Support for this work from the 21C Frontier Microbial Genomics and Applications Center Program (M102KK010011-08K1101-01110), Ministry of Education, Science and Technology, Republic of Korea, to H.-G. Hur and the Korea Research Foundation (MOEHRD) (KRF-2007-357-D00141), Ministry of Education, Science and Technology, Republic of Korea, to J.-H. Lee is gratefully acknowledged.

REFERENCES

1. **Demergasso, C. S., C. D. Guillermo, E. G. Lorena, J. J. Pueyo Mur, and C. Pedrós-Alió.** 2007. Microbial precipitation of arsenic sulfides in Andean salt flats. Geomicrobiol. J. **24:**111–123.
2. **Ledbetter, R. N., S. A. Connon, A. L. Neal, A. Dohnalkova, and T. S. Magnuson.** 2007. Biogenic mineral production by a novel arsenic-metabolizing thermophilic bacterium from the Alvord Basin, Oregon. Appl. Environ. Microbiol. **73:**5928–5936.
3. **Lee, J. H., J. H. Han, H. C. Choi, and H. G. Hur.** 2007. Effects of temperature and dissolved oxygen on Se(IV) removal and Se(0) precipitation by *Shewanella* sp. HN-41. Chemosphere **68:**1898–1905.
4. **Lee, J. H., M. G. Kim, B. Yoo, N. V. Myung, J. Maeng, T. Lee, A. C. Dohnalkova, J. K. Fredrickson, M. J. Sadowsky, and H. G. Hur.** 2007. Biogenic formation of photoactive arsenic-sulfide nanotubes by *Shewanella* sp. strain HN-41. Proc. Natl. Acad. Sci. USA **104:**20410–20415.
5. **Lee, J. H., Y. Roh, K. W. Kim, and H. G. Hur.** 2007. Organic acid-dependent iron mineral formation by a newly isolated iron-reducing bacterium, *Shewanella* sp. HN-41. Geomicrobiol. J. **24:**31–41.
6. **Macy, J. M., J. M. Santini, B. V. Pauling, A. H. O'Neill, and L. I. Sly.** 2000. Two new arsenate/sulfate-reducing bacteria: mechanisms of arsenate reduction. Arch. Microbiol. **173:**49–57.
7. **Newman, D. K., T. J. Beveridge, and F. M. M. Morel.** 1997. Precipitation of arsenic trisulfide by *Desulfotomaculum auripigmentum*. Appl. Environ. Microbiol. **63:**2022–2028.
8. **Newman, D. K., E. K. Kennedy, J. D. Coates, D. Ahmann, D. J. Ellis, D. R. Lovley, and F. M. M. Morel.** 1997. Dissimilatory arsenate and sulfate reduction in *Desulfotomaculum auripigmentum*, sp. nov. Arch. Microbiol. **165:**380–388.
9. **Oremland, R. S., and J. F. Stolz.** 2005. Arsenic, microbes and contaminated aquifers. Trends Microbiol. **13:**45–49.
10. **Saltikov, C. W., A. Cifuentes, K. Venkateswaran, and D. K. Newman.** 2003. The *ars* detoxification system is advantageous but not required for As(V) respiration by the genetically tractable *Shewanella* species strain ANA-3. Appl. Environ. Microbiol. **69:**2800–2809.
11. **Saltikov, C. W., and D. K. Newman.** 2003. Genetic identification of a respiratory arsenate reductase. Proc. Natl. Acad. Sci. USA **100:**10983–10988.
12. **Saltikov, C. W., R. J. Wildman, and D. K. Newman.** 2005. Expression dynamics of arsenic respiration and detoxification in *Shewanella* sp. strain ANA-3. J. Bacteriol. **187:**7390–7396.
13. **Silver, S.** 1998. Genes for all metals—a bacterial view of the periodic table. J. Ind. Microbiol. Biotechnol. **20:**1–12.
14. **Stolz, J. F., P. Basu, J. M. Santini, and R. S. Oremland.** 2006. Arsenic and selenium in microbial metabolism. Annu. Rev. Microbiol. **60:**107–130.
15. **Stolz, J. F., and R. S. Oremland.** 1999. Bacterial respiration of arsenic and selenium. FEMS Microbiol. Rev. **23:**615–627.