Reduction of Uranium(VI) to Uranium(IV) by Clostridia[▽]

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Several different species of clostridia reduced U(VI) to U(IV) to various degrees. The optimal pH for U(VI) reduction is 5 to 6 in most cases; a *Clostridium* sp. showed the highest rate at pH 4. Nitrate did not affect U(VI) reduction, indicating that this process in clostridia is nitrate independent.

Previously, we showed that a *Clostridium* sp. (ATCC 53464) and *Clostridium sphenoides* (ATCC 19403) can reduce uranyl-nitrate, -acetate, and -citrate complexes as well as complexes of other metals, implying that the reduction of uranyl compounds is a general property of this genus (4–6). Apparently, *Clostridium* is one of major players in uranium reduction in situ in acidic (pH 4) uranium mine pit water (11, 13), at a military facility near Chesapeake Bay, MD (1), and at the ORFRC at Oak Ridge, TN (8). In this study, we investigated the abilities of several strains of clostridia to reduce uranium(VI) under fermentative conditions, viz., the *Clostridium* sp. (ATCC 53464) that we isolated in our laboratory and *C. sphenoides* (ATCC 19403), *Clostridium acetobutylicum* (ATCC 824), and *Clostridium pasteurianum* (ATCC 7040).

All strains were grown anaerobically in mineral salts medium (4) containing glucose, but only *C. sphenoides* also metabolized citric acid. The fermentation products, including organic acids and gases (hydrogen and carbon dioxide), were analyzed by high-performance liquid chromatography and gas chromatography. Analysis of culture medium showed that all the strains produced acetic acid and butyric acid. To stabilize the pH of the culture as needed, we added 50 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); pH 6.8] or 50 mM MES (morpholineethanesulfonic acid; pH 6.2) to the medium. We removed 4-milliliter aliquots of the culture and measured change in pH and turbidity at 600 nm. Total protein was determined by using a Micro Lowry total protein kit (Sigma) (10).

Ten milliliters of the culture at the late log phase was transferred to an anaerobic, preautoclaved 20-ml serum bottle, and then 0.1 ml of a 10 mM U(VI)-nitrate solution prepared by dissolving $UO_2(NO_3)_2$ in prereduced deionized water was added via a 1-ml syringe. To determine the U(VI) concentration over time, aliquots of 0.1 to 0.2 ml of the bacterial culture were taken from the serum bottle, and 5 μl of the culture was diluted in 2 ml deionized water and immediately analyzed for U(VI) by a kinetic phosphorescence analyzer (KPA-11A; Chemchek Instruments, Inc.). To assess the effect of pH on U(VI) reduction by clostridia, 10-milliliter aliquots of the bacterial culture at the late log growth phase were adjusted to the required pH values by using 1 N NaOH or HCl.

The culture sample remaining in the serum bottle after sampling for the uranium reduction assay was transferred to a centrifuge tube in the glove box. After its pH was adjusted to 11 with 1 N NaOH to precipitate the uranium, the culture was centrifuged at 10,000 rpm for 10 min, the supernatant discarded, and the pellet resuspended in 2 ml of a 10 mM citric acid solution to extract the uranium species. The solution was filtered through a 0.45-μm membrane filter, and the filtrate was analyzed by UV-visible light (UV-vis) spectrophotometry to determine the absorption spectra of uranium(VI) and -(IV).

Figure 1 shows the reductions of U(VI) by clostridia at the various pHs and the respective UV-vis spectra for U(VI) (major absorption peak at 438 nm) and U(IV) (major and minor peaks at 662 nm and 560 nm, respectively). Reduction of U(VI) by the *Clostridium* sp. was observed at pH 3.0, 4.2, and 5.2. The rate of reduction was much faster at pH 4.2 than at pH 3 or 5.2. No reduction of U was observed at pH 1.3. In contrast, *C. acetobutylicum* showed a marked reduction of uranium(VI) at pH 5.8 and 4.7; at both pHs, the U(IV) peaks at both 662 and 560 nm became very pronounced. At pH 3.2, little U(VI) reduction was observed and only a U(VI) peak was visible. *C. pasteurianum* exhibited a moderate reduction of U(VI) to U(IV) at pH 5.8, and even at this pH, only the 662-nm peak of U(IV) was observed. Maximum reduction of U(VI) by *C. sphenoides* occurred at pH 6.5, and lower pHs affected the reduction. These results suggest that by and large that uranium reduction at higher pHs is more favorable for strains other than the *Clostridium* sp. strain (Fig. 1).

We determined the effect of U(VI) concentration on U reduction by the *Clostridium* sp. The bacterium reduced 0.6 mM U(VI) at the maximum concentration tested (Fig. 2A). The reduction of U(VI) proceeded at the same rate for about 6 h, followed by lower rates at all U(VI) concentrations (Fig. 2A). We monitored the reduction of U(VI) up to 72 h and found that not all uranium, especially at higher concentrations, was completely reduced to U(IV) (Fig. 2B). Furthermore, the increased concentration of nitrate derived from the added U(VI) source did not affect U(VI) reduction, indicating that this process in the *Clostridium* sp. is nitrate independent, a characteristic applicable to clostridia and not found in many other anaerobic respiratory bacteria, such as those of the genera *Desulfovibrio*, *Geobacter*, and *Shewanella*.

The rates of reduction of 0.1 mM of U(VI) at different pHs by clostridia are shown in Fig. 3. The optimum rates of reduc-
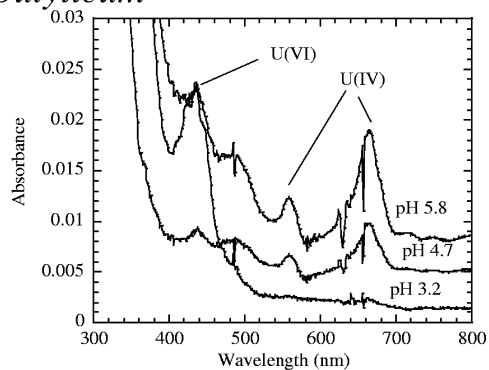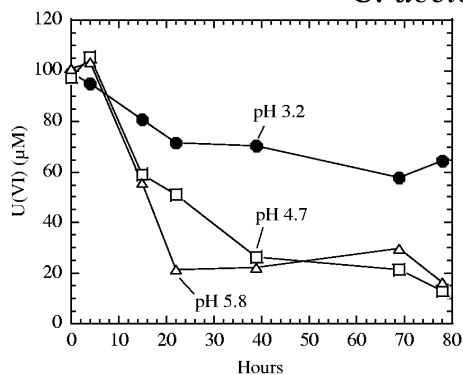
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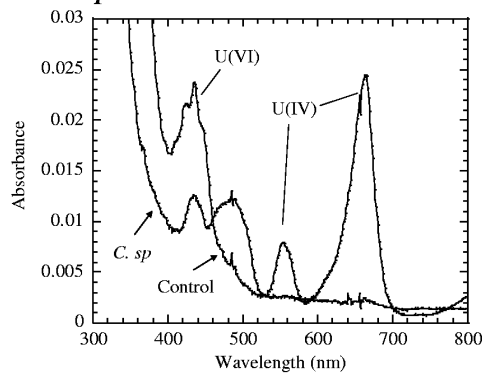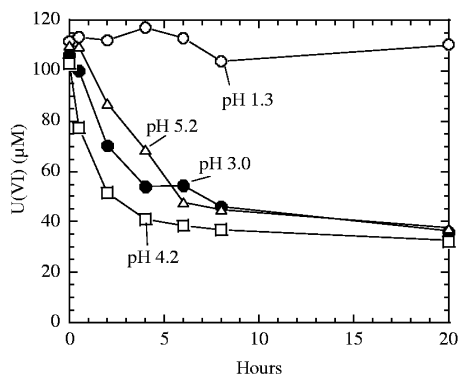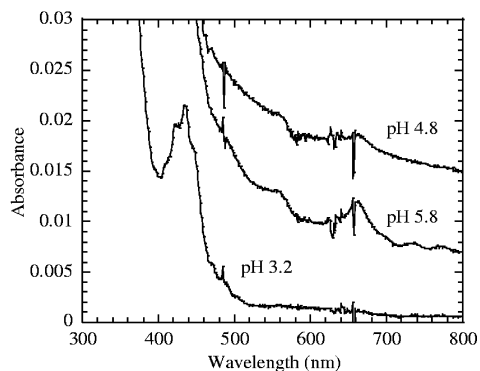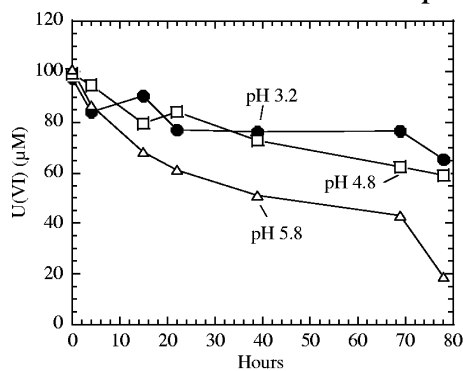
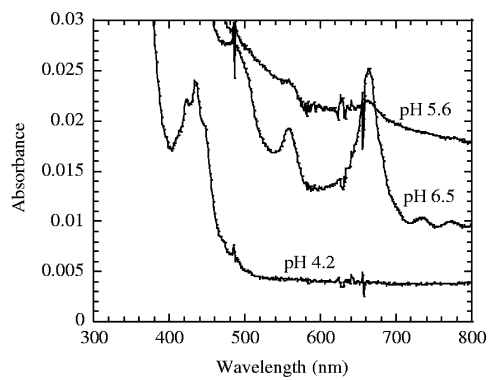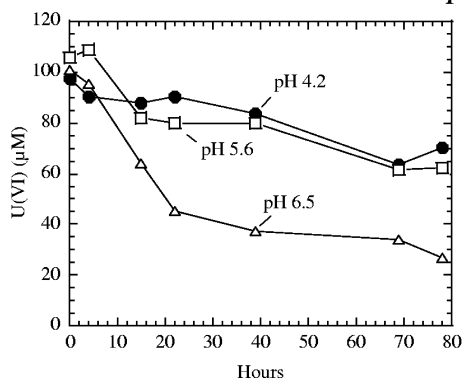FIG. 1. Reduction of U(VI) by clostridia and UV-vis spectra of U(VI) and U(IV) at different pHs. The initial pHs of cultures subjected to the reduction assay are given. All strains were cultured in mineral salts medium.
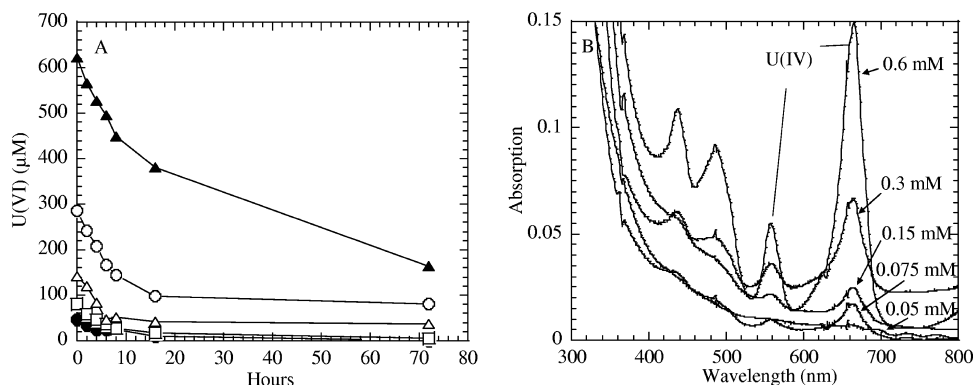
FIG. 2. Reduction of U(VI) by the *Clostridium* sp. at different initial U(VI) concentrations. Uranium was added as $UO_2(NO_3)_2$. The initial optical density at 600 nm of bacteria was 0.5. The U(VI) concentration is noted.

tion of U(VI) (μM h^{-1} g^{-1} protein) were as follows: *Clostridium* sp., 83.4 at pH 4.2; *C. acetobutylicum*, 12.4 at pH 5.8; *C. pasteurianum*, 7.2 at pH 5.8; and *C. sphenoides*, 31.3 at pH 6.5. Thus, the *Clostridium* sp. showed the highest U(VI) reduction, at a rate of 83.4 μM U(VI) h^{-1} g^{-1} protein at pH 4, a value that is about 200%, 400%, and 800% higher than those for *C. sphenoides*, *C. acetobutylicum*, and *C. pasteurianum*, respectively, at their optimal pHs of 5.8, 5.8, and 6.5.

We identified that the *Clostridium* sp. was the closest phenotypically to *C. pasteurianum* (unpublished data). Like *C. pasteurianum*, the *Clostridium* sp. can fix nitrogen. By a partial 16S rRNA gene sequencing, we obtained further evidence verifying their phylogeny affiliations. The total DNAs of strains were isolated as described previously (20). The 16S rRNA genes were amplified from isolates with the PCR primers FD1 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1540R (5′-AAG GAG GTG ATC CAG CC-3′) (17, 19). The DNA sequences were determined with a BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems), using a 3700 DNA analyzer (Perkin-Elmer) and the primer 529R (5′-CGC

GGC TGC TGG CAC-3′) (19). We compared the obtained sequences against those in GenBank through the NCBI Internet web service and aligned them with ClustalW (15). Phylogenetic analyses of 16S rRNA gene sequences were undertaken using MEGA version 4.0 (14). A phylogeny tree was constructed by a neighbor-joining algorithm under a ρ distance model, with the bootstrap computed 500 times. Based on a DNA sequence of about 400 bp, we constructed a phylogeny tree containing all the strains used in this study and some other clostridia (Fig. 4). This phylogeny tree shows that only *C. sphenoides* is classified into group A, while all other three strains that we explored fall into group B.

The phylogeny analysis agreed with a previous phenotypic analysis showing that the *Clostridium* sp. is closest to *C. pasteurianum*. The partial 16S rRNA sequence of the *Clostridium* sp. is 98% identical to that of *C. pasteurianum*. Nevertheless, the difference between the two is evident in terms of uranium reduction. Similarly, although *Clostridium butyricum* is phylogenetically related to a novel electrochemically active and Fe(III)-reducing bacterium, it does not itself necessarily possess the same properties (9). Given the plasticity of the bacterial genome, which is subject to frequent recombination and reconstruction (12), it is not surprising to observe differences in some characteristics of the *Clostridium* sp. and *C. pasteurianum*.

With their widespread occurrence in soils, sediments, and low-level radioactive wastes, clostridia could play a significant role in the in situ reduction of uranium and other metals, particularly at acidic pHs and in nitrate-rich environments (1, 2, 7, 8, 11, 13). We found that all of the clostridia tested not only reduced uranium but differed in extent of reduction and that the pH of the medium strongly influences the reduction. The *Clostridium* sp., which was isolated from an acidic metal-contaminated site (3), has higher tolerance to harsh environmental conditions, such as low pH, and a stronger ability for fermentation and reduction of U(VI) and other metals in acidic environments.

Although the mechanisms of U(VI) reduction by *Desulfovibrio*, *Geobacter*, and *Shewanella* species are known (16, 18), the mechanisms of uranium reduction by clostridia remain unclear. Our previous study showed that it is an enzymatic process, since it occurred only in the presence of growing or
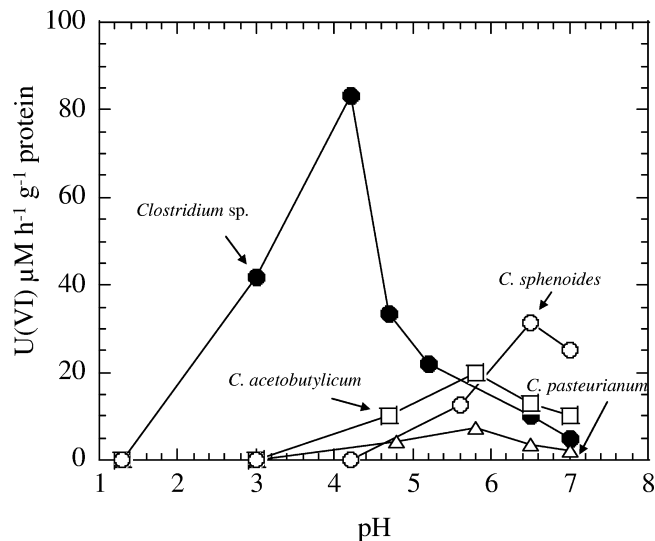

FIG. 3. Rates of reduction of U(VI) added as uranyl nitrate by clostridia at different pHs.
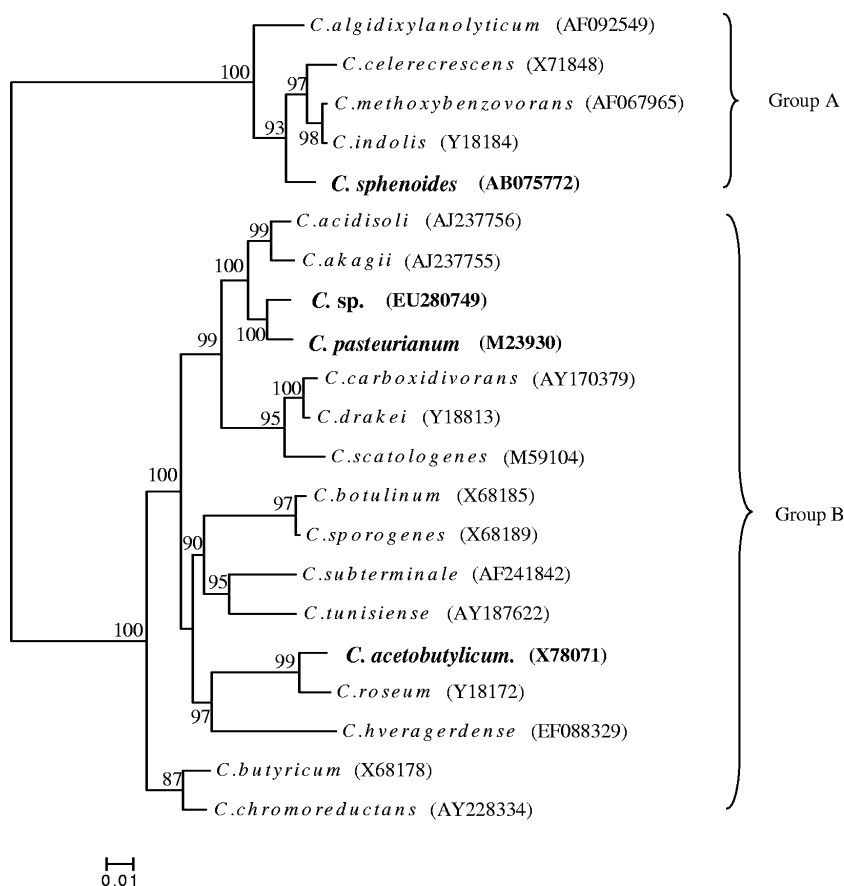
FIG. 4. Phylogenetic relationships between strains used in this study (in bold) and other clostridia. Bootstrap ($n = 500$) values above 50 are shown. GenBank accession numbers are in parentheses.

resting cells; neither the organic acid metabolites generated, the extracellular components of the culture, nor heat-killed cells could reduce uranium anaerobically (4). Other conjectures were that the reducing power generated from glucose fermentation caused uranium reduction. Thus, Petrie at al. (11) demonstrated that glucose amendment to the growth medium enriched the numbers of gram-positive, spore-forming bacteria and, since some of the highest rates of U(VI) reduction in situ occurred upon amendment, that fermentative processes were involved (7). Our results herein show that optimizing the conditions for fermentation resulted in better U(VI) reduction, supporting this hypothesis.

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