Utilization of DNA as a Sole Source of Phosphorus, Carbon, and Energy by *Shewanella* spp.: Ecological and Physiological Implications for Dissimilatory Metal Reduction[▽]

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The solubility of orthophosphate (PO_4^{3-}) in iron-rich sediments can be exceedingly low, limiting the bioavailability of this essential nutrient to microbial populations that catalyze critical biogeochemical reactions. Here we demonstrate that dissolved extracellular DNA can serve as a sole source of phosphorus, as well as carbon and energy, for metal-reducing bacteria of the genus *Shewanella*. *Shewanella oneidensis* MR-1, *Shewanella putrefaciens* CN32, and *Shewanella* sp. strain W3-18-1 all grew with DNA but displayed different growth rates. W3-18-1 exhibited the highest growth rate with DNA. While strain W3-18-1 displayed Ca^{2+}-independent DNA utilization, both CN32 and MR-1 required millimolar concentrations of Ca^{2+} for growth with DNA. For *S. oneidensis* MR-1, the utilization of DNA as a sole source of phosphorus is linked to the activities of extracellular phosphatase(s) and a Ca^{2+}-dependent nuclease(s), which are regulated by phosphorus availability. Mass spectrometry analysis of the extracellular proteome of MR-1 identified one putative endonuclease (SO1844), a predicted UshA (bifunctional UDP-sugar hydrolase/5′ nucleotidase), a predicted PhoX (calcium-activated alkaline phosphatase), and a predicted CpdB (bifunctional 2′,3′ cyclic nucleotide 2′ phosphodiesterase/3′ nucleotidase), all of which could play important roles in the extracellular degradation of DNA under phosphorus-limiting conditions. Overall, the results of this study suggest that the ability to use exogenous DNA as the sole source of phosphorus is widespread among the shewanellae, and perhaps among all prokaryotes, and may be especially important for nutrient cycling in metal-reducing environments.

Phosphorus (P) is a key element that often limits bacterial growth in various freshwater and marine habitats (13, 40, 44, 51, 53). Inorganic phosphate (P_i), or orthophosphate (PO_4^{3-}), can serve as a direct source of P for essentially all physiological groups of microorganisms in both natural environments and laboratory media. Measurements of soluble phosphate in different aquatic environments, however, suggest that concentrations of bioavailable P_i are very low. Recent studies have revealed that P_i represents only a small fraction of soluble reactive P in natural waters and that even in eutrophic systems, its concentration may be as low as 27 pM (2, 20, 25). This is not surprising, since in a variety of aquatic systems, soils, and sediments, P_i bioavailability can be controlled by adsorption to metal oxides (3, 16, 59) and through chemical reactions with hydrous oxides, amorphous and crystalline complexes of Fe, Al, and Ca, and organic matter (2, 41, 49). In particular, the reaction of P_i with Fe(III) oxides such as goethite can result in the precipitation of tinticite [$Fe_6(PO_4)_4(OH)_6 \cdot 7H_2O$] (22) or griphite [$Fe_3Mn_2(PO_4)_2 \cdot 5(OH)_2$] (32) depending on the solution and surface conditions. The latter findings entail important physiological implications for dissimilatory metal-reducing bacteria residing in zones with high concentrations of Fe(III) and Mn(III, IV) oxides, where the levels of bioavailable P_i may

significantly limit growth. In that respect, utilization of other bioavailable forms of P may be the key to the ecological success of these bacteria.

Alternative sources of P in freshwater and marine environments are organic phosphorus compounds that include phospholipids, phosphoproteins, and nucleic acids. It has been demonstrated that dissolved extracellular DNA constitutes a significant portion of the organic P pool, where DNA concentrations can range from 0.2 to 88 μg/liter (0.65 to 280 nM P) (7, 24, 36, 43). The main sources of dissolved nucleic acids in aquatic environments include phage-induced cell lysis and excretion by zooplankton feeding on bacteria (48, 52, 55). Earlier studies showed that the addition of DNA to natural water samples resulted in its degradation and incorporation into cells and sometimes in an increase in bacterial cell numbers (17, 23, 34, 42, 55).

To gain a better understanding of physiological and mechanistic aspects of microbial nucleic acid utilization by dissimilatory metal-reducing organisms, we investigated the abilities of *Shewanella* spp. to utilize DNA for growth and respiration. Physiological and biochemical measurements were carried out using *Shewanella oneidensis* MR-1, *Shewanella putrefaciens* CN32, and *Shewanella* sp. strain W-3-18-1, isolated from freshwater, terrestrial, and marine environments, respectively, to allow a broader representation of the various ecophysiotypes within the genus. Overall, the results indicate that DNA could be used as a sole source of carbon, nitrogen, and P by all three strains under aerobic and anaerobic conditions and could serve as an electron donor for metal reduction. Application of con-

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tinuous-cultivation, proteomic, and biochemical methods suggested that the ability of *Shewanella* to utilize dissolved nucleic acids as the sole source of P depends, in part or in whole, on the extracellular phosphatase activities and, under certain conditions, on the extracellular nuclease activities.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *S. oneidensis* MR-1 (54), *S. putrefaciens* CN-32 (16), and *Shewanella* sp. strain W3-18-1 (45) were routinely cultured aerobically at 30°C with shaking at 150 rpm using tryptic soy broth (TSB) or M1 minimal medium (pH 7.0) containing (per liter) 30 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES), 28 mM NH$_4$Cl, 30 mM NaCl, 3 mM MgCl$_2$ · 6H$_2$O, 1.34 mM KCl, 6.8 µM CaCl$_2$, 1 µM Na$_2$SeO$_4$, and 10 ml each of 10× Wolfe's vitamin solution and 10× mineral solution (26). When necessary, supplemental CaCl$_2$ was added to the M1 medium to a final concentration of 1 mM. Either NaH$_2$PO$_4$ · H$_2$O or DNA was used as a source of P. All growth experiments were performed using low-molecular-weight (average, 200 bp, as estimated by agarose gel electrophoresis) herring sperm DNA. For batch cultivation, the M1 medium was supplemented with 30 mM D,L-sodium lactate or 1 g/liter DNA as the sole source of carbon and energy. The modified M1 medium used for continuous cultivation contained 3 mM PIPES, 18 mM D,L-sodium lactate, 1 mM CaCl$_2$, 10 µM ferric nitrilotriacetic acid [Fe(III)-NTA], and different concentrations of NaH$_2$PO$_4$ · H$_2$O or DNA as a source of P. For anaerobic growth, the M1 medium was supplemented with 10 mM Fe(III)-NTA as an electron acceptor. The medium was purged with O$_2$-free N$_2$, anaerobically dispensed into N$_2$-flushed Balch tubes or serum bottles, sealed with butyl rubber stoppers, and sterilized by autoclaving.

For DNA-dependent growth with lactate as the source of carbon and energy, DNA was added to the phosphate-free medium to final concentrations ranging from 20 to 300 mg/liter. The DNA was sterilized by filtering through a 0.22-µm-pore-size membrane or was added to the medium prior to autoclaving. To obtain phosphorus-starved *Shewanella* cultures, phosphorus-free M1 medium containing 30 mM sodium lactate was inoculated with a 0.4% (vol/vol) overnight-grown TSB culture and was incubated for 48 h. To ensure that no residual P remained in the cultures, a second transfer into the same medium was performed. The cultures were considered phosphorus starved if no growth occurred after the second transfer. These phosphorus-starved cultures were used as inocula for growth experiments unless stated otherwise. All growth experiments were independently repeated at least twice and performed in triplicate.

Chemostat cultivation. All chemostat experiments were performed using 6-liter New Brunswick Bioflow 3000 reactors (New Brunswick Scientific, Edison, NJ) operated at a 3-liter working volume at 30°C. Reactors were inoculated with 120-ml phosphorus-starved *S. oneidensis* MR-1 cultures and were maintained in a batch mode until late-logarithmic stage. The continuous mode was initiated and maintained at a dilution rate of 0.088 h^{-1}. The gas flow rate and agitation were kept at 4 liters/min and 450 rpm, respectively, and the dissolved oxygen concentration was maintained at 5% of air saturation by changing the ratio of N$_2$ and air in the gas mix. The pH was maintained at 7.0 by automatic addition of 2 M HCl. Periodically, 5- to 10-ml samples were taken from the reactors for further analyses.

DNA preparations. Lyophilized herring sperm DNA was purchased from Sigma (St. Louis, MO; catalog no. D3159). Stock solutions were prepared by dissolving the DNA in M1 minimal medium (pH 7.0) immediately before use. For nuclease activity assays, we used a 1,526-bp PCR product that had been amplified from the pDS1779 plasmid using the M13 forward primer (5'-GTA AAA CGA CGG CCA G-3') and reverse primer (5'-CAG GAA ACA GCT ATG AC-3') and *Taq* polymerase. The pDS1779 plasmid consisted of two fragments of the *S. oneidensis* MR-1 genome (bases 1861034 to 1861707 and bases 1863885 to 1864489 from GenBank accession number NC_004347) that had been linked via a 20-bp linker (AGA GAC GAC CTA AGC CAG TA) and cloned into XcmI-digested pDS3.1 (54a). Following PCR amplification, the product was purified using a QIAquick PCR purification kit (Qiagen Inc., Valencia, CA) and was quantified spectrophotometrically by absorbance at 260 nm.

Preparation of extracellular proteins and proteome analysis. For extracellular proteome analysis, 1-liter samples of chemostat grown MR-1 cultures were centrifuged at 9,200 × g (10 min, 4°C) and the resulting supernatant was passed through a 0.22-µm-pore-size membrane filter (Nalgene, Rochester, NY). The supernatant was further concentrated 100-fold by ultrafiltration through 5-kDa-cutoff Ultracel cellulose filters (Millipore, Billerica, MA) using Amicon Stirred Cell 8400. Concentrated protein samples were digested with 2 µg of modified porcine trypsin (Promega, Madison, WI). The resulting peptide samples were

subjected to liquid chromatography-tandem mass spectrometry (LC–MS-MS) using an LCQ Deca XP^{PLUS} ion-trap mass spectrometer (Thermo-Electron, San Jose, CA). Each sample was centrifuged at 16,000 × g for 15 min prior to dilution to 0.2 µg/µl with 1% acetic acid in distilled H$_2$O, and 2 µg (10 µl) of each peptide sample was loaded by a Famos autosampler (Dionex, Sunnyvale, CA) onto a hand-packed reverse-phase C$_{18}$ high-performance LC (HPLC) column for each LC–MS-MS experiment. The samples were subjected to 120-min HPLC (HP-1100; Agilent, Palo Alto, CA) gradients (5% plus 80% acetic acid in distilled H$_2$O against 100% acetonitrile); the LC-MS setup has been described elsewhere (28). The mass spectrometer was operated in the data-dependent acquisition mode using Xcalibur (version 1.4) software. The experiment consisted of a single full-scan mass spectrum (400 to 2,000 m/z) followed by three data-dependent MS-MS scans. The database search program TurboSEQUEST (version 27, revision 12) (12) was used to identify peptides in the MS-MS spectra. Searches of spectra at charge states +1, +2, and +3 were conducted using the following search parameters: no enzyme specificity; peptide mass tolerance, 2.5; average molecular weight for both peptide and fragment ion masses; a b/y ion search including neutral losses from b/y ions; and variable modification at M = +15.995. The SEQUEST output was processed by the LIPS (logistic identification of peptide sequences) model (19) to generate peptide identification probabilities. Three or more peptides with probabilities above 0.95 were used for positive identification. Orthologs in other *Shewanella* spp. were identified using the BLAST programs provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and Gnare (Genome Analysis Research Environment; http://compbio.mcs.anl.gov/gnare/gnare_home.cgi). Genome neighborhood analysis was conducted using the Integrated Microbial Genomes environment (IMG; http://img.jgi.doe.gov/cgi-bin/pub/main.cgi).

Analytical methods. Nuclease activity was assayed by filtering the culture supernatant through a 0.22-µm-pore-size Millex-GP membrane filter (Millipore) and then mixing a 230-µl aliquot of this filtrate with 20 µl of 1,526-bp PCR-amplified DNA (final concentration, 2 µg/ml). The reaction mixtures were typically incubated for 60 min at 30°C, and 25-µl aliquots were removed timewise from the reaction mixture. The DNA was visualized by agarose gel electrophoresis. If no DNA was visible on a gel, it was assumed that all DNA was digested. The nuclease activity was expressed as the amount of DNA digested per milliliter of filtered culture liquid per min.

Phosphatase activity was assayed by mixing 0.9 ml of 0.22-µm-pore-size-membrane-filtered culture supernatant with 0.1 ml of 100 mM p-nitrophenylphosphate (final concentration, 10 mM) and monitoring 4-nitrophenol accumulation by the change in the optical density at 418 nm (OD$_{418}$) at 30°C with a Shimadzu UV-2101PC scanning spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). The slope of the OD$_{418}$ change for the first 5 min (linear range) was taken to determine the phosphatase activity by using a calibration curve prepared with 4-nitrophenol.

Inorganic phosphate concentrations were measured using the previously established molybdate technique (35). The iron reduction rates were measured colorimetrically using the capture reagent ferrozine, which forms a chromophoric complex with Fe(II) that is detectable at 562 nm (47). To measure the total Fe, samples were treated with hydroxylamine hydrochloride to reduce Fe(III) to Fe(II) prior to analysis by the ferrozine assay (15). Cell numbers in experiments with Fe(III)-NTA as the electron acceptor were determined by acridine orange direct epifluorescent microscopic counting. Organic acids were quantified using an HP 1090 HPLC with a diode array detector at 210 nm. The mobile phase was 0.005 N H$_2$SO$_4$ at 0.5 ml/min, and the column was a 300- by 78-mm Rezex organic acid column (Phenomenex, Torrance, CA) at 60°C.

RESULTS

***Shewanella* grows under aerobic and anaerobic conditions with DNA as a sole source of P.** To probe the mechanisms of P assimilation by dissimilatory metal-reducing bacteria, we examined the aerobic growth of three *Shewanella* isolates, *S. oneidensis* MR-1, *S. putrefaciens* CN32, and *Shewanella* sp. strain W3-18-1, with lactate as the source of carbon and energy and DNA as the sole source of P. A series of experiments indicated that DNA supported vigorous aerobic growth of all three strains (Fig. 1). The growth rate and culture turbidity (OD$_{600}$) differed for each strain, with W3-18-1 showing the fastest growth on both phosphate and DNA and having no preference for one P source over the other. By contrast, CN32
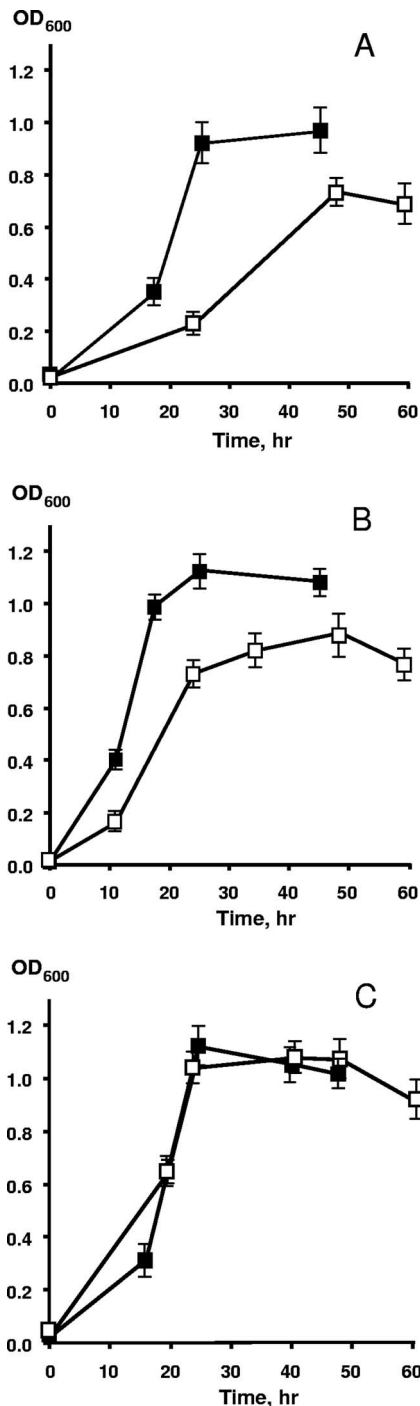
FIG. 1. Aerobic growth of *S. oneidensis* MR-1 (A), *S. putrefaciens* CN32 (B), and *Shewanella* sp. strain W3-18-1 (C) with NaH_2PO_4 (■) or DNA (□) as the sole source of phosphorus. Approximately 5% of phosphorus-starved cultures were used as inocula for all strains. The DNA and $NaH_2PO_4 \cdot H_2O$ concentrations in M1 medium were 300 mg/liter and 133.6 mg/liter, respectively, to achieve a final P concentration of 30 mg/liter. DNA was sterilized by autoclaving with the medium. The time course for growth was inferred from the OD_{600}. The data points and error bars represent the means and standard deviations of triplicate cultures, respectively.
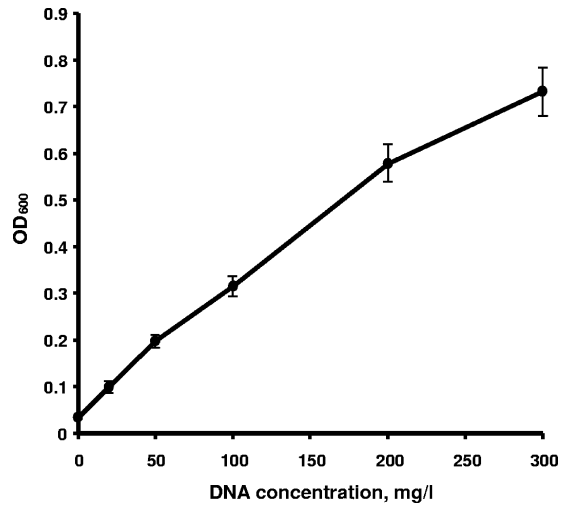

FIG. 2. Relationship between *S. oneidensis* MR-1 aerobic growth for 48 h and initial DNA concentrations. DNA was added to the medium before autoclaving and served as the sole P source. Five percent of phosphorus-starved cultures were used as inocula.

and MR-1 achieved consistently higher growth rates and final cell densities when $NaH_2PO_4 \cdot H_2O$ was provided as the sole P source. As shown for MR-1, the increase in cell density during aerobic growth was nearly linear with increasing DNA concentrations (Fig. 2). Similar results were obtained for CN32 and W3-18-1 (data not shown). When DNA was autoclaved with the medium, the aerobic cultures of *S. oneidensis* MR-1 displayed higher growth yields, exceeding the cell densities observed with filter-sterilized DNA by 30 to 80%. This observation is consistent with the notion that autoclaving may cause partial DNA hydrolysis and is supported by our results showing that some DNA degradation to smaller fragments occurred as a result of autoclaving (data not shown), although free phosphate levels were still below detection (<0.25 μM).

Next, we examined the abilities of three *Shewanella* strains to grow by reducing Fe(III)-NTA under anaerobic conditions using filter-sterilized DNA as a sole source of P and lactate as the electron donor. The ability to use DNA was assessed by five consecutive serial transfers (a 20-fold dilution factor was used) to fresh medium to minimize nutrient carryover from the inoculum. In each transfer, 90 to 98% of the total Fe(III) was reduced in 30 to 50 h. No formation of a secondary Fe(II) phase was observed during reduction by any of the three strains. Quantitative measurements indicate that within 20 h, in the presence of DNA as the sole source of P, *S. oneidensis* MR-1 reached the maximum cell count and achieved nearly complete reduction of Fe(III), while there was no growth and relatively slow reduction without any P source (Fig. 3). No significant differences in Fe(III) reduction rates were observed between cultures using DNA or NaH_2PO_4. The time to reach maximum cell density was longer with NaH_2PO_4 for reasons that are unclear.

Extracellular phosphatase activity is detected in *S. oneidensis* MR-1 culture filtrates. To gain a basic understanding of the possible mechanisms of DNA utilization, we assayed extracellular nuclease and phosphatase activities in 0.22-μm-pore-size-filtered samples of *S. oneidensis* MR-1 cultures grown in the
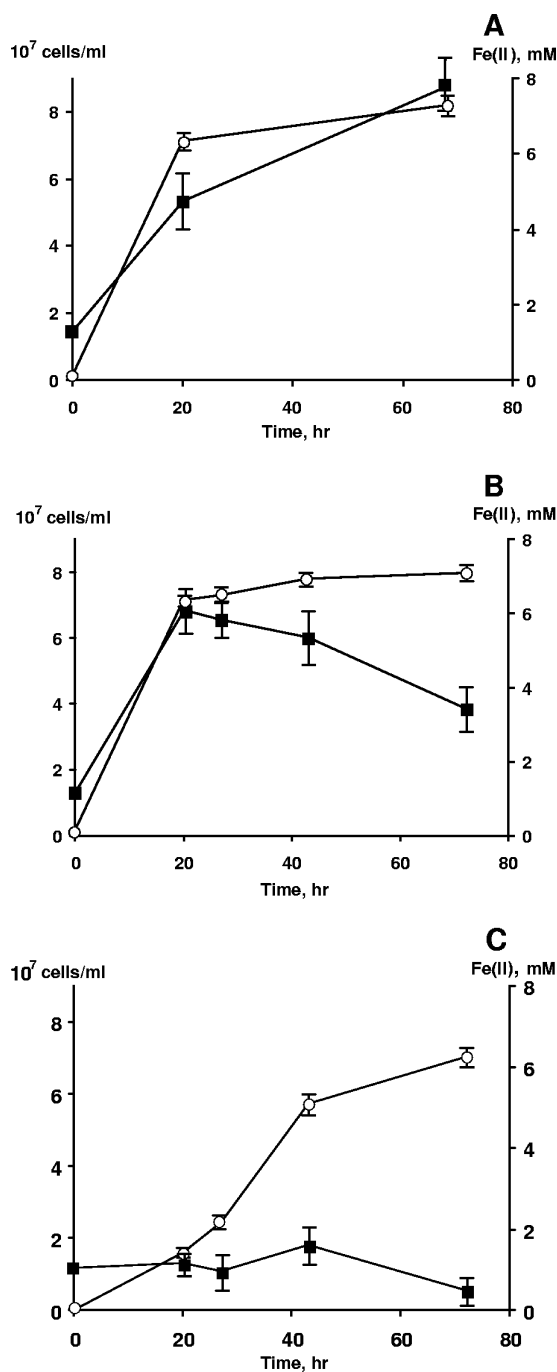
FIG. 3. Growth of *S. oneidensis* MR-1 in phosphate-free M1 medium with 8 mM Fe(III)-NTA as the electron acceptor and 30 mM lactate as the electron donor and carbon source. (A and B) $NaH_2PO_4 \cdot H_2O$ (A) and DNA (B) were used at concentrations of 60 mg/liter and 100 mg/liter, respectively. Filter-sterilized DNA was added to the medium after autoclaving. (C) Growth and Fe(III) reduction in the absence of a P source. ■, cell numbers; ○, Fe^{2+} concentrations.

presence of DNA. Initial assays failed to detect cell-free nuclease activity in any of the MR-1 cultures grown with 150 or 300 mg of DNA/liter. In addition, agarose gel electrophoresis of cell supernatants revealed that the added DNA remained intact (data not shown), despite apparent utilization of P.

In contrast, phosphatase activity measured with *p*-nitrophenylphosphate reached 0.6 μmol/ml/min in cultures grown with 150 mg/liter of DNA for 48 h. To determine whether P_i is released as a result of extracellular phosphatase activity, we added DNA at final concentrations of 300 mg/liter to DNA-grown MR-1 culture filtrates (pore size, 0.22 μm) and incubated the mixture for 30 min at 30°C using a heat-inactivated filtrate as a negative control. Addition of a molybdate-based reagent for a P (PO_4) assay (35) led to time-dependent development of a characteristic blue color due to the formation of a P-molybdate complex in the experimental tubes, whereas the controls remained colorless. Unfortunately, quantitative measurements of P (PO_4) formation were not possible due to the formation of a precipitate in both the control and the experimental samples. We interpret these results as evidence for the production by *S. oneidensis* MR-1 of an extracellular phosphatase that is able to cleave phosphate groups from the 5′ end of DNA.

Effect of Ca^{2+} on DNA utilization by different *Shewanella* strains. Although the growth of *S. oneidensis* MR-1 with DNA as a sole source of P was not accompanied by DNA hydrolysis and MR-1 displayed no extracellular nuclease activity, its genome encodes two proteins, SO1844 and SO1066, that belong to the COG2374 predicted extracellular-nuclease group (for complete genome sequence information, see http://www.ncbi.nlm.nih.gov) (18). Because some extracellular nucleases require divalent cations to be functional (4, 14, 31, 33, 38), we reasoned that the inability of MR-1 to cleave DNA molecules could be a result of the relatively low Ca^{2+} concentration (6.8 μM) in M1 growth medium. To address this question, we first examined the effect of $CaCl_2$ on DNA assimilation by the three *Shewanella* strains. Addition of 1 mM $CaCl_2$ greatly enhanced the ability of MR-1 (Fig. 4A) and CN32 (data not shown) to utilize DNA as the sole source of P for growth. In contrast, *Shewanella* sp. strain W3-18-1 grew equally well with either DNA or NaH_2PO_4, and additions of $CaCl_2$ had no effect on growth kinetics (Fig. 4B), suggesting that DNA hydrolysis by this particular strain is not Ca^{2+} dependent.

Analysis of MR-1 culture filtrates indicated that no residual extracellular DNA was detected after 48 h of incubation in the presence of 1 mM $CaCl_2$. Additions of 1 mM Ca^{2+} to MR-1 cultures grown aerobically in the presence of 50 mg DNA/liter resulted in threefold biomass increases as indicated by optical density measurements (Fig. 4A). In contrast, when NaH_2PO_4 was provided as the sole P source, the addition of 1 mM $CaCl_2$ did not stimulate growth (data not shown). Taken together, these results suggest that added exogenous DNA may be digested by a Ca^{2+}-dependent nuclease(s) produced by growing cultures of MR-1 and CN32. Nevertheless, 0.22-μm-pore-size filtrates prepared from MR-1 cultures grown with DNA in the presence of 1 mM $CaCl_2$ failed to exhibit nuclease activity (data not shown).

Growth studies and enzyme activities in continuous cultures of *S. oneidensis* MR-1 under phosphorus and carbon limitation. Previous investigations have indicated that P availability can have a profound effect on the phosphorus assimilation pathways, since starvation or limitation may induce the expression of extracellular nuclease activities (21, 50). Although the lack of nuclease activity in batch culture supernatants could be the result of cell-bound enzymatic action, we hypothesized that
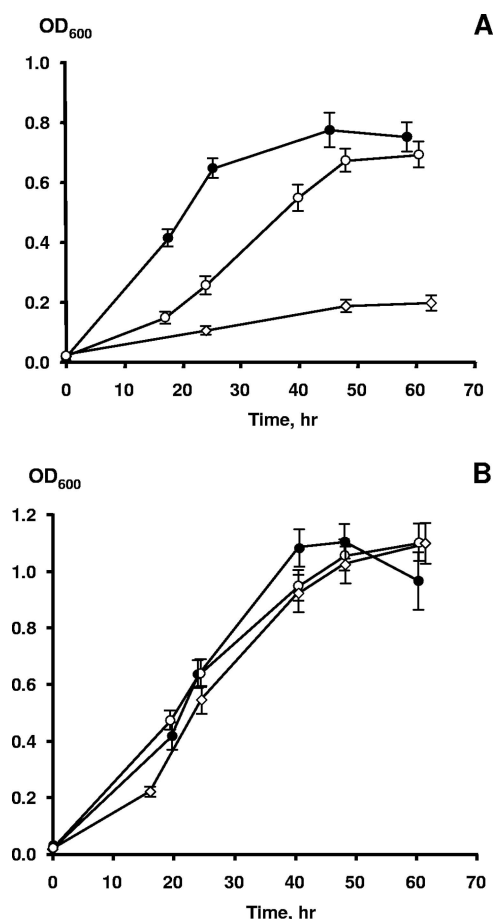
FIG. 4. Aerobic growth of *S. oneidensis* MR-1 (A) and *Shewanella* sp. strain W3-18-1 (B) with NaH$_2$PO$_4$ (●), DNA without supplemental CaCl$_2$ (◇), or DNA with 1 mM CaCl$_2$ (○) as the sole source of phosphorus. Approximately 5% of phosphorus-starved cultures were used as inocula for all strains. The DNA and NaH$_2$PO$_4$ · H$_2$O concentrations in M1 medium were 50 mg/liter and 22.3 mg/liter, respectively, to achieve a final P concentration of 5 mg/liter. The time course for growth was inferred from the OD$_{600}$. The data points and error bars represent the means and standard deviations of triplicate cultures, respectively.

the development of extracellular nuclease activity may also have been suppressed by the excess of DNA or products of its degradation in the medium. To investigate this, *S. oneidensis* MR-1 was grown in continuous culture with variable concentrations of P (0.1 mM, 0.5 mM, and 1.5 mM), which included

PO$_4^{3-}$ or DNA in the presence of 1 mM CaCl$_2$. Our results indicated that steady-state cultures grown with 0.1 mM P (PO$_4$) or DNA were in fact P limited, as shown by the presence of acetate, the product of incomplete lactate oxidation, in the supernatant. Moreover, the cell densities in these cultures were about one-half of those obtained under carbon limitation with 1.5 mM P, and the concentrations of free P (PO$_4$) in filtered supernatants were below 0.25 μM (Table 1). Nuclease and phosphatase assays using culture filtrates from both PO$_4$ and DNA continuous cultures grown in the presence of 0.1 mM P indicated apparent time-dependent increases in the specific activities of both enzymes (Fig. 5A and C). In particular, nuclease activity increased 4- to 12-fold in DNA and PO$_4$ reactors, respectively, after ~7 reactor volume changes.

Elevating the P concentration to 0.5 mM resulted in a twofold increase in biomass, while the acetate concentrations declined below detection levels in culture filtrates. The P limitation was relieved in these cultures; the residual P (PO$_4$) concentrations in both DNA- and PO$_4$-supplied reactors increased to 1.12 μM and 2.27 μM, respectively. At 0.5 mM P, no extracellular nuclease activity was detected in the PO$_4$ reactor, while the extracellular nuclease activity in the DNA-fed reactor decreased eightfold from that under P-limited conditions (Table 1). Increasing the P concentration further to 1.5 mM did not result in significant increases in cell density in either reactor. The extracellular nuclease activity in the DNA reactor decreased below detection levels, while free DNA was present in the culture filtrates (data not shown). Similarly, the concentrations of P (PO$_4$) increased to 0.8 mM in filtrates from the PO$_4$ continuous culture. Cell-free phosphatase activities in both the PO$_4$ and DNA reactors decreased twofold with an increase in P concentration from 0.1 to 0.5 mM. Further increasing the P concentration to 1.5 mM did not affect phosphatase activity in the DNA culture, whereas in the PO$_4$ reactor, the extracellular phosphatase activity decreased nearly 10-fold (Table 1).

It should be noted that P availability had a profound effect on the behavior of aerobic chemostat cultures of *S. oneidensis* MR-1, resulting in an intriguing physiological manifestation. In earlier studies, we have shown that aerobically grown MR-1 forms tight multicellular aggregates in the presence of millimolar concentrations of Ca^{2+} (J. S. McLean et al., unpublished data). The aggregates dissipate upon addition of EDTA, presumably due to the complexation of Ca^{2+}, which serves as a bridging cation, resulting in an increase in optical density. Therefore, EDTA was used to estimate the biomass concen-

TABLE 1. Biochemical and physiological parameters of *S. oneidensis* MR-1 growth in chemostat cultures[a]

Concn of phosphorus in medium (mM)	OD$_{600}$		Residual acetate concn[b] (mM)		P (PO$_4$) concn in fermentor (μM)		Extracellular nuclease activity (μg/min/ml)		Extracellular phosphatase activity (nmol/min/ml)	
	PO$_4$	DNA	PO$_4$	DNA	PO$_4$	DNA	PO$_4$	DNA	PO$_4$	DNA
0.1	0.355	0.355	4.67	4.76	BD	BD	0.8	0.8	5.5	5.3
0.5	0.735	0.759	BD	BD	2.27	1.12	BD	0.1	2.4	2.1
1.5	0.780	0.755	BD	BD	800	ND	BD	BD	0.34	2.2

[a] Reactors were supplemented with NaH$_2$PO$_4$ or DNA as a P source (designated "PO$_4$" and "DNA," respectively). BD, below detection; ND, not detected.
[b] Measured in the reactors after steady state was achieved. The concentrations of lactate and pyruvate were always below detection levels.
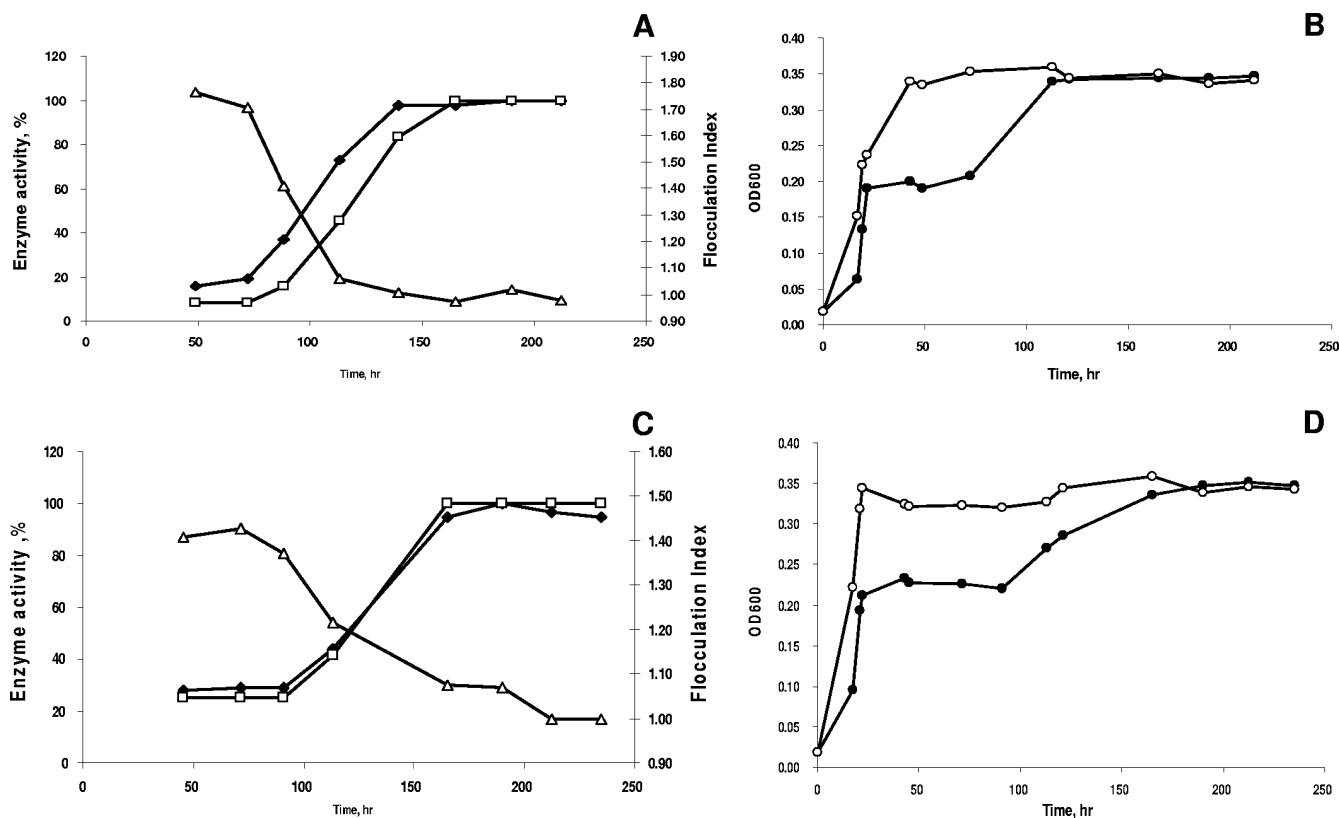
FIG. 5. Growth and extracellular phosphatase and nuclease activities in chemostat cultures of MR-1 in the presence of NaH_2PO_4 (A and B) and DNA (C and D). The flocculation index ($\triangle$) was calculated based on the optical densities of cultures measured without ($\bullet$) and with ($\bigcirc$) 10 mM EDTA. Extracellular phosphatase ($\blacklozenge$) and nuclease ($\square$) activities in chemostat cultures of *S. oneidensis* MR-1 were measured as described in Materials and Methods.

tration of aggregated cultures. The ratio of OD_{600} values with and without 10 mM EDTA was designated the flocculation index (FI) and reflected the extent of aggregation. In this study, FI stability was one of the parameters used to define steady-state conditions in aerobic P-limited cultures. Our observations indicate that the aggregation of aerobic *S. oneidensis* MR-1 cultures was usually stable for at least 7 reactor volume changes when *S. oneidensis* was cultured aerobically in a carbon-limited chemostat with 1.5 mM NaH_2PO_4 in the presence of 1 mM $CaCl_2$. Under these conditions, no extracellular nuclease activity was detected (Table 1). Under P limitation, however, the aerobic chemostat cultures of MR-1 gradually disaggregated, as reflected by the decrease in the FI. Our results show that the dissipation of cell aggregates coincided with a significant increase in extracellular nuclease and phosphatase specific activities (Fig. 5A to D).

Extracellular proteome analysis of P-limited chemostat cultures. To investigate the identity of proteins that may contribute to the extracellular nuclease and phosphatase activities in MR-1, we isolated total extracellular proteins from a P (PO_4)-limited chemostat culture and subjected them to LC–MS-MS analysis. Measurements of total extracellular protein concentrations showed 2.5 to 3.0 mg/liter under steady-state conditions when the P concentration was 0.1 mM. Table 2 contains a list of proteins that were detected with high confidence in filtered chemostat samples of *S. oneidensis* MR-1 grown under

these conditions. Out of 22 proteins, all but 2, purine nucleoside phosphorylase (SO1221) and cysteine synthase A (SO2903), were predicted to be localized to the outside of the cytoplasmic membrane, indicating the absence of any significant cell lysis. Notably, the predicted functions of seven proteins (SO0990, SO1221, SO1560, SO1844, SO2001, SO2385, and SO3565) were related to P or DNA/nucleotide metabolism. Among these, SO1844 is predicted to encode an endonuclease and could account for the DNase activity observed in the supernatants of cells grown on Na_2HPO_4. The protein encoded by SO2385, originally annotated as a hypothetical protein, shares 42% identity with the recently discovered PhoX monomeric alkaline phosphatase of *Pasteurella multocida* (57). Both SO2385 and PhoX possess an N-terminal leader peptide that is characteristic of lipoproteins secreted across the cytoplasmic membrane by the twin arginine translocation system. PhoX is activated by calcium, suggesting that the increased growth of MR-1 on DNA observed in calcium-supplemented medium is due in part to activation of the phosphatase activity encoded by SO2385.

We have also identified two putative proteins, SO2001 and SO3565, that can catalyze the hydrolysis of 2′,3′ cyclic phosphates of adenosine, guanosine, cytosine, and uridine with the formation of a free phosphate group. SO3565 encodes a bifunctional 2′,3′ cyclic nucleotide 2′ phosphodiesterase/3′ nucleotidase similar to the CpdB protein in *Escherichia coli*.

TABLE 2. List of extracellular proteins identified in filtered supernatants of MR-1 chemostat cultures grown with 0.1 mM P (PO$_4$)

Locus tag[a]	Functional annotation	Predicted localization	Peptide count[b]
SO0189	Outer membrane fibronectin type III/cadherin domain protein	Outer membrane; β-barrel protein	3
SO0990	Periplasmic amidohydrolase 3 family protein	Periplasm	12
SO1221	Purine nucleoside phosphorylase (DeoD)	Cytoplasm	4
SO1557	Outer membrane porin	Outer membrane; β-barrel protein	20
SO1560	Phosphate-binding protein	Periplasm	23
SO1779	Outer membrane decaheme cytochrome *c* (OmcA)	Outer membrane; lipoprotein	5
SO1822	TonB-dependent receptor	Outer membrane; β-barrel protein	11
SO1844	Outer membrane endonuclease/exonuclease/phosphatase	Outer membrane; β-barrel protein	82
SO2001	Bifunctional UDP-sugar hydrolase/5′ nucleotidase (UshA)	Outer membrane; lipoprotein	30
SO2385	Monomeric alkaline phosphatase (PhoX)	Extracellular	35
SO2903	Cysteine synthase A (CysK)	Cytoplasm	8
SO2907	TonB-dependent receptor	Outer membrane; β-barrel protein	14
SO3237	Flagellin (FliC1)	Extracellular; flagella	22
SO3238	Flagellin (FliC2)	Extracellular; flagella	5
SO3247	Flagellar hook protein (FlgE)	Extracellular; flagella	11
SO3545	Outer membrane porin	Outer membrane; β-barrel protein	4
SO3565	Bifunctional 2′,3′ cyclic nucleotide 2′ phosphodiesterase/3′ nucleotidase (CpdB)	Outer membrane; lipoprotein	9
SO3896	Outer membrane porin (Omp35)	Outer membrane; β-barrel protein	8
SO4317	Cell surface calcium-binding protein with bacterial neuraminidase repeats	Extracellular	11
SO4652	ABC sulfate/thiosulfate transporter, periplasmic ligand-binding subunit (Sbp)	Periplasm	16
SO4719	ABC tungstate transporter, periplasmic ligand-binding subunit (TupA)	Periplasm	8
SOA0110	Expressed lipoprotein	Outer membrane; lipoprotein	5

[a] Locus tag numbers correspond to the NCBI *S. oneidensis* MR-1 genome annotation (http://www.ncbi.nlm.nih.gov).
[b] Number of peptides detected for a particular protein. Only nonoverlapping, fully tryptic peptides are included.

However, unlike the corresponding proteins in *E. coli* and other bacteria, which are predicted to be periplasmic, the putative CpdB from MR-1 is predicted to be localized to the outer membrane. Similarly, SO2001, which encodes a putative UDP-sugar hydrolase (UshA) protein, contains the N- and C-terminal 5′ nucleotidase domains. Furthermore, like CpdB, the *Shewanella* UshA proteins are predicted to be lipoproteins, while their counterparts in enteric bacteria are predicted to be periplasmic. Recent studies with *Corynebacterium glutamicum* showed that UshA is synthesized and secreted into the medium during phosphate starvation, suggesting involvement in the utilization of nucleotides and related compounds as sources of phosphorus (39). Collectively, the proteomic data indicate that *S. oneidensis* MR-1 excretes a number of extracellular proteins with putative functions in DNA degradation as a result of P limitation. Moreover, the extracellular nuclease and phosphatase activities and possibly nuclease localization are regulated by the P source and, most likely, P (PO$_4$), availability.

***Shewanella* can use DNA as a sole source of carbon, energy, phosphorus, and nitrogen.** *Shewanella* strains are commonly isolated from freshwater or marine sediments and the water column, where DNA may be abundant (6, 7, 24, 36, 43). Given the results of our preliminary experiments demonstrating that some members of a bacterial population isolated from the upper layer of the Columbia River (Washington State) were able to grow with DNA as the sole nutrient, and the fact that MR-1 is able to use adenosine as a sole source of carbon and energy (M. Driscoll, unpublished data), we hypothesized that *Shewanella* can use DNA as a sole source of carbon and energy.

The results of our experiments presented in Fig. 6 show that all three strains were able to grow aerobically with DNA as the only C, N, P, and energy source for at least four transfers. Strain W3-18-1 showed the highest growth rates and final OD$_{600}$, whereas MR-1 grew significantly more slowly (Fig. 6A and B). These results strongly suggest that these *Shewanella* strains are able to obtain nitrogen by catabolizing one or more of the nucleotides present in DNA. Anaerobic growth with filter-sterilized DNA as the sole nutrient was also examined in the presence of Fe(III)-NTA as the electron acceptor. Altogether, six consecutive serial transfers (using a 10-fold dilution factor) were performed; strains CN32 and W3-18-1 reduced 90% to 99% of Fe(III) in a period from 100 to 114 h upon each transfer. MR-1 also grew and reduced Fe(III) with DNA but required two to three times longer to achieve the same extent of reduction (data not shown). The Fe(III) reduction rates are consistent with the slower aerobic growth of MR-1 using DNA as the sole nutrient (Fig. 6). Hence, growth with DNA as the sole source of carbon, energy, nitrogen, and phosphorus under both aerobic and anaerobic conditions appears to be a common trait of metal-reducing *Shewanella* spp.

DISCUSSION

The presence of extracellular DNA in water columns and sediments has been well documented, with concentrations often exceeding those of inorganic phosphate (2, 7, 20, 24, 25, 36, 43). DNA, as well as RNA and protein, associated with dead or moribund cells and insoluble organic matter settles through the water column in freshwater and marine environments and eventually reaches sediments, where it can potentially be assimilated by bacteria, including metal-reducing species. Anal-
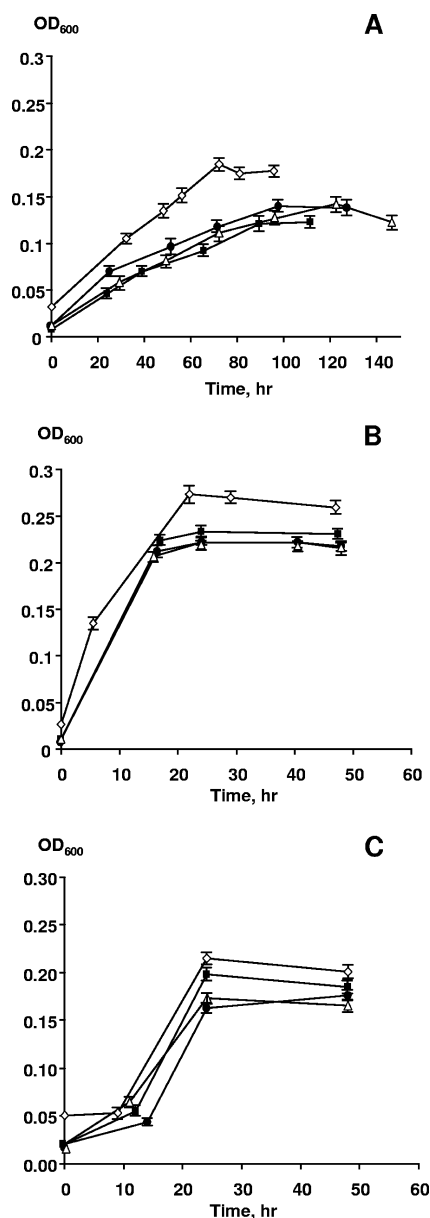
FIG. 6. Aerobic growth of *S. oneidensis* MR-1 (A), *Shewanella* sp. strain W3-18-1 (B), and *S. putrefaciens* CN32 (C) with 1 g/liter of DNA as the sole source of carbon, energy, N, and P in phosphate- and nitrogen-free M1 medium. The inoculum consisted of a P-starved culture that was diluted 20-fold into M1 medium. The time course for growth was inferred from the OD_{600}. The data points and error bars represent the means and standard deviations of triplicate cultures, respectively. $\Diamond$, first transfer; ■, second transfer; $\triangle$, third transfer; ●, fourth transfer.

yses of marine sediments have shown that polymeric carbohydrates, proteins, and DNA may constitute most of the organic matter, because soluble and labile organic compounds are consumed by bacteria before particles reach the sea floor (5, 8, 30). Moreover, extracellular DNase activity was found in both sediments and seawater (8, 9, 30), suggesting ongoing DNA degradation. The ability to use DNA as a sole source of P may be especially beneficial for metal-reducing bacteria such as *Sh-*

ewanella, since phosphate complexation with Fe(II) or sorption to Fe(III) oxides (16, 22, 59) may limit P_i bioavailability.

In this work, we tested the abilities of three strains of *Shewanella*, *S. oneidensis* MR-1, *S. putrefaciens* CN32, and *Shewanella* sp. strain W3-18-1, isolated from freshwater lake sediment, the subsurface, and marine sediment, respectively, to use DNA as a sole source of P, and we probed for mechanisms involved in the utilization of this organic polymer. While their growth rates and requirements for Ca^{2+} differed, all three strains were capable of utilizing DNA not only as a P source but also as a sole source of nitrogen, carbon, and energy to maintain growth under both aerobic and anaerobic conditions. Of the three strains tested, *S. oneidensis* MR-1 showed the lowest rate of growth with DNA as a sole nutrient both aerobically and anaerobically. The reason for this is unclear, although the ability to slowly use DNA as an energy source may be beneficial for long-term survival when other carbon and electron sources in sediments are exhausted.

Using MR-1 as an example, we showed that the ability of *Shewanella* to utilize DNA as a P source was linked to extracellular nuclease and phosphatase activities. Interestingly, extracellular nuclease activity was absent in filtrates from batch cultures of *S. oneidensis* grown with DNA. On the one hand, it is likely that DNA utilization in batch cultures is a result of cell-bound nuclease activity. On the other hand, the development of extracellular nuclease activity could be repressed by high initial DNA concentrations and the accumulation of degradation products. To alleviate the limitations of batch cultivation, we grew MR-1 in chemostats to impose specific nutrient limitations. The results from these experiments indicated that P limitation promoted extracellular nuclease and phosphatase activities, either by increasing the expression levels or by releasing preexisting proteins to the extracellular milieu. Higher extracellular phosphatase and nuclease activities were measured when either P_i or DNA was the growth-limiting substrate in the chemostat cultures, suggesting that the specific activities of these enzymes were independent of the P source.

Chemostat cultivation of MR-1 under P limitation resulted in another interesting observation: dissipation of oxygen- and Ca^{2+}-induced cell aggregation, which coincided with an increase in extracellular nuclease activity. Extracellular DNA has been identified in a number of biofilm systems (37, 46, 56) and appears to play a role as a structural component. Addition of DNase I to *Pseudomonas aeruginosa* PAO1 biofilms was reported to disaggregate the colonies (56). By analogy, we reasoned that extracellular DNA could be present in the MR-1 aggregate matrix and that its depolymerization by extracellular nuclease resulted in culture disaggregation. To evaluate this hypothesis, the MR-1 aggregates were investigated using the cell membrane-impermeant DNA stain 7-hydroxy-9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one) (DDAO) as described in references 1 and 58 and the membrane-permeant DNA stain Syto 59. The confocal images indicate that extracellular DNA is in fact present throughout the structure and can be clearly seen in association with the intracellular matrix (Fig. 7).

Interestingly, W3-18-1, which is the only marine isolate among the three *Shewanella* strains used in this study, displayed Ca^{2+}-independent utilization of DNA, whereas both CN32 and MR-1, which were isolated from the subsurface and freshwater sediments, respectively, depended on Ca^{2+} to fully
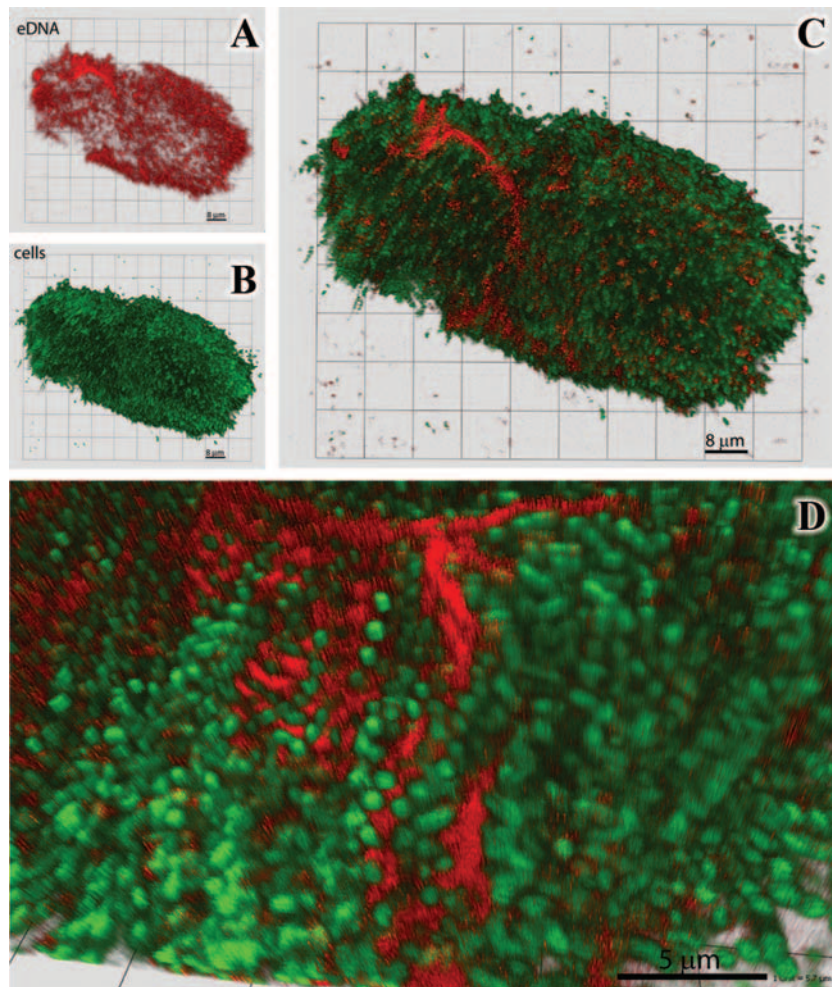
FIG. 7. Representative 3-dimensional confocal images of the MR-1 multicellular aggregates. (A to C) Differential staining using fluorescent dyes for extracellular DNA (DDAO; red) (A) and intercellular DNA (Syto 59; green) (B) and a merged image (C) reveal the spatial distribution and relative volumetric percentages of cells and matrix material. (D) Cells (green) and DDAO stain (red) at high resolution show extracellular DNA staining between cells.

digest DNA. From an ecological standpoint, this observation is somewhat counterintuitive, given that concentrations of Ca^{2+} in seawater are typically significantly higher than those in freshwater (27). Nonetheless, it indicates fundamental differences in the enzyme repertoire or DNA utilization pathways displayed by different strains of *Shewanella*. To probe this question, we investigated whether the genome sequences of strains CN32 and W3-18-1 contain any homologs of MR-1 genes SO1844 and SO1066, both of which belong to the COG2374 family of extracellular nucleases. Comparative analysis of the CN32 and W3-18-1 genomes indicated that both strains encode only two members of the COG2347 family orthologous to SO1066 and SO1844, of which only the latter was detected in supernatants of P-limited MR-1 cultures. While no unique known nucleases are found in the genome of W3-18-1, it is feasible to suggest that this bacterium encodes unknown novel Ca^{2+}-independent extracellular nucleases.

It is also possible that the nuclease genes in W3-18-1 are regulated differently from those in MR-1 and that their expression under specific growth conditions contributes to Ca^{+2}-

independent utilization of DNA. In working with *Shewanella* strain W3-18-1, we have observed that this strain does not form biofilms on glass surfaces, nor does it form aggregates in the presence of Ca^{2+} (J. S. McLean, unpublished data). Previously, it was shown that the locus encoding a type I secretion system and the putative SO4317 adhesin detected in our P-limited supernatants are associated with biofilm and aggregate formation in MR-1 (11). In *Shewanella* sp. strain W3-18-1, however, the ATPase/C39 family cysteine peptidase component (Sputw3181_3731) of the analogous type I secretion system is interrupted by an insertion element. Consequently, we hypothesize that this strain is unable to secrete the SO4317 ortholog (Sputw3181_3730), leading to an inability to form biofilms or aggregate in the presence of Ca^{2+}. Further evidence of biofilm formation deficiency in W3-18-1 is provided by the observation that three of the O-antigen biosynthesis genes are pseudogenes (Sputw3181_1473, -1475, and -1478) and that the putative *pilN* gene, encoding a type IV pilin biogenesis protein, is interrupted by an insertion element. Given this evidence, it is reasonable to propose that the loss of

many functions associated with biofilm behavior in W3-18-1 may be balanced by changes in regulatory networks, leading to expression of different genes (e.g., the SO1066-type nuclease) in the absence of Ca^{2+} during growth as cell suspensions.

In light of earlier studies by Dell'Anno and Danovaro (10), which suggested that almost 50% of the P demand of deep-sea prokaryotic populations worldwide is supplied by DNA, the implications of the current work may be expanded to different environments. Indeed, independent experiments confirmed that the metal-reducing bacterium *Geobacter sulfurreducens* and the gram-positive bacterium *Bacillus subtilis* were able to use DNA as the sole source of P, while the prokaryotic population residing in the upper layer of the Columbia River grew rapidly in river water supplemented with 1 g of DNA/liter (unpublished data). Furthermore, the recently published results on DNA utilization by different groups of marine bacteria (29) support the notion that the ability to use exogenous DNA as a source of phosphorus, carbon, energy, and nitrogen is widespread among some groups of prokaryotes and may be especially important for nutrient cycling in PO_4-limited environments in which metal-reducing bacteria reside.

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