Regulation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) metabolism in *Shewanella halifaxensis* HAW-EB4 by terminal electron acceptor and involvement of c-type cytochrome

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Shewanella halifaxensis HAW-EB4 was previously isolated for its potential to mineralize hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) from a UXO (unexploded ordnance)-contaminated marine sediment site near Halifax Harbor. The present study was undertaken to determine the effect of terminal electron acceptors (TEA) on the growth of strain HAW-EB4 and on the enzymic processes involved in RDX metabolism. The results showed that aerobic conditions were optimal for bacterial growth, but that anaerobic conditions in the presence of trimethylamine *N*-oxide (TMAO) or in the absence of TEA favoured RDX metabolism. RDX as a substrate neither stimulated respiratory growth nor induced its own biotransformation. Strain HAW-EB4 used periplasmic proteins to transform RDX to both nitroso [hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX)] and ring cleavage products (such as methylenedinitramine), with more nitroso formation in cells grown on TMAO or pre-incubated in the absence of TEA. Using spectroscopy, SDS-PAGE and haem-staining analysis, strain HAW-EB4 was found to produce different sets of c-type cytochromes when grown on various TEA, with several more cytochromes produced in cells grown on TMAO. Crude cytochromes from total periplasmic proteins of TMAO-grown cells metabolized RDX to products similar to those found in assays using total periplasmic proteins and whole cells. To prove the involvement of cytochrome in RDX metabolism, we monitored dithionite- or NADH-reduced cytochromes by their absorbance at the α (551 nm) or γ (418–420 nm) bands during anaerobic incubation with RDX. In both cases we found that RDX biotransformation was accompanied by oxidation of reduced cytochrome. Furthermore, O₂, an oxidant of reduced cytochrome, inhibited RDX transformation. The present results demonstrate that *S. halifaxensis* HAW-EB4 metabolizes RDX optimally under TMAO-reducing conditions, and that c-type cytochromes are involved.

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INTRODUCTION

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a widely used military explosive and a toxic pollutant that currently contaminates many military sites (EPA, 1988; Lotufo *et al.*, 2001; Robidoux *et al.*, 2002). Both aerobic and anaerobic bacteria have been found to be capable of biotransforming RDX (Zhao *et al.*, 2004a; Lewis *et al.*, 2004; Crocker *et al.*, 2006). Previously, strains of *Shewanella* were isolated from an unexploded ordnance (UXO)-contaminated marine

sediment site near Halifax Harbor for their potential to mineralize RDX under oxygen-limiting conditions via the intermediary formation of nitroso derivatives and ring cleavage products (Fig. 1; Zhao *et al.*, 2004b). These strains were also found to represent novel species of *Shewanella*, including *Shewanella halifaxensis* and *Shewanella sediminis* (Zhao *et al.*, 2005, 2006). Recently, the genomes of *S. halifaxensis* HAW-EB4 and *S. sediminis* HAW-EB3 were sequenced to help determine how RDX is degraded in the environment.

Shewanella are bacteria that primarily live in the marine environment, and are known for their ability to grow aerobically and anaerobically using a wide range of terminal electron acceptors (TEA), such as nitrate and trimethylamine *N*-oxide (TMAO) (Bowman, 2005). Nitrate (Kamykowski & Zentara, 1985; Kaspar, 1982; Jørgensen,

Abbreviations: DNX, hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine; MEDINA, methylenedinitramine; MNX, hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine; NDAB, 4-nitro-2,4-diazabutanol; RDX, hexahydro-1,3,5-trinitro-1,3,5-triazine; TEA, terminal electron acceptor(s); TMAO, trimethylamine *N*-oxide; TMBZ, 3,3,5,5-tetramethylbenzidine; TNX, hexahydro-1,3,5-trinitroso-1,3,5-triazine; UXO, unexploded ordnance.

Fig. 1. RDX metabolic routes in anaerobic bacteria modified from pathways previously published for *Klebsiella pneumoniae* strain SCZ-1 (Zhao *et al.*, 2002), *Clostridium bifermentans* strain HAW-1 (Zhao *et al.*, 2003) and diaphorase (Bhushan *et al.*, 2002).

1989; Prospero & Savoie, 1989; Sayama, 2001) and TMAO (Yancey *et al.*, 1982; Kelly & Yancey, 1999) are abundant in marine sediments; therefore, they are expected to influence *in situ* growth of *Shewanella*, including *S. halifaxensis* HAW-EB4 in the marine sediment from which this bacterium was isolated. Despite recent progress made toward understanding RDX degradation, little information is available on the effect of TEA on the expression of RDX metabolic activity in anaerobic bacteria (Kitts *et al.*, 2000) such as *Shewanella* and on the type of enzymes involved in RDX degradation. The goal of the present study was to determine the effect of TEA on RDX metabolic activity in *S. halifaxensis* HAW-EB4 and the potential involvement of *c*-type cytochrome in RDX reduction.

METHODS

Chemicals and reagents. RDX (99 % pure) and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX; 99 % pure) were provided by Defense Research and Development Canada (DRDC), Quebec, Canada. Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX; 98 % pure), methylenedinitramine (MEDINA), 4-nitro-2,4-diazabutanal (NDAB; 99 % pure), and a mixture of MNX (23 %, w/w), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX; 56 %, w/w) and TNX (21 %, w/w), were purchased from SRI International. All other chemicals, including sodium nitrate and TMAO, were reagent grade.

Growth of *S. halifaxensis* HAW-EB4. *S. halifaxensis* HAW-EB4 (=NCIMB 14093=DSM 17350) was routinely grown at 10 °C under aerobic conditions, either on solid Brewer anaerobic agar containing 4 % (w/v) sea salts (Sigma) or in marine broth 20 medium (MB 20) composed of Bacto peptone (1.6 %, w/v), yeast extract (0.4 %, w/v) and sea salts (4 %, w/v). MB 20 was also used as a basal medium for growth of strain HAW-EB4 under aerobic, O_2 -limiting, or anaerobic

conditions in the presence of nitrate or TMAO, or in the absence of any TEA. Growth in liquid medium was conducted on a rotary shaker at 150 r.p.m. and 10 °C, unless otherwise noted. Aerobic growth was conducted in shaken, foam-plugged 1 l Erlenmeyer flasks containing 150 ml medium. Growth under O_2 -limiting conditions was conducted in static, foam-plugged 37.5 ml serum bottles containing 20 ml medium. Anaerobic growth was conducted in sealed serum bottles (37.5 ml) containing 20 ml medium in the presence of nitrate or TMAO, or in the absence of any TEA. TMAO or sodium nitrate was added to the medium from a sterile stock solution (1.1 M, prepared in MB 20 medium before use) to the desired concentrations. Prior to inoculation, the liquid media were made anaerobic by degassing, followed by charging with argon three times. The inocula (10 %, v/v) were prepared by growing strain HAW-EB4 in MB 20 medium aerobically for 24 h to reach an OD_{600} close to 4.

RDX metabolism by growing or resting cells of strain HAW-EB4. RDX degradation by growing cells (initial OD_{600} 0.1) of strain HAW-EB4 was conducted by incubating the bacterium in MB 20 medium with RDX (20 mg l^{-1}) under aerobic, O_2 -limiting or anaerobic (in the presence of 5 mM sodium nitrate or TMAO, or in the absence of TEA) conditions.

RDX metabolism by resting cells was carried out using the following protocols. Briefly, cells of strain HAW-EB4 were harvested by centrifugation at 11 000 g, 4 °C for 20 min after 12.5 h incubation (initial OD_{600} 0.38) either in 150 ml aerobic medium (in a 1 l flask) or in 650 ml (2×500 ml serum bottles, each containing 325 ml medium) O_2 -limiting or anaerobic medium, using the above conditions. The final OD_{600} and the wet weight of biomass after harvest were [condition/ OD_{600} /wet weight of cells (g)]: aerobic/1.88/1.5; O_2 -limiting/0.44/0.94; nitrate (50 mM)-reducing/0.54/1.7; TMAO (100 mM)/0.85/1.8; absence of any TEA/0.42/0.94. Cells were resuspended in varying volumes of anaerobic sea salt (4 %) solution to give an OD_{600} of 8.1 (wet weight $34 \pm 6 \text{ g l}^{-1}$). RDX biotransformation mixtures were prepared by mixing 8.5 ml of the above-mentioned cell suspensions with 11.5 ml RDX (33.8 mg l^{-1} ,

sterilized by filtering through a 0.22 µm pore-size membrane filter) sea salt stock solution in 20 ml serum bottles (final cell density 15 ± 2 g wet weight l^{-1}). Afterwards, the serum bottles were sealed and made anaerobic by briefly degassing (1 min), followed by recharging with argon three times. RDX biotransformation was conducted at 10 °C in the dark under static incubation conditions. Aerobic reactions were conducted in foam-plugged serum bottles on a rotary shaker at 150 r.p.m. and 10 °C. An abiotic control containing a sterile sea salts solution of RDX without bacteria was prepared. Sea salts (4%, w/v) solution used for resuspending the cells was made anaerobic prior to use by repeated degassing (10–20 min), followed by recharging with argon three times.

Preparation of spheroplast and periplasmic proteins.

Spheroplasts and periplasmic proteins were prepared by the subcellular fractionation method of Birdsell & Cota-Robles (1967) as modified by Easter *et al.* (1983). This method has been widely used for subcellular fractionation of *Shewanella*, including *Shewanella frigidimarina* NAMB400 (previously classified as *Alteromonas* sp. NCMB 400; Easter *et al.*, 1983; Reid & Gordon, 1999; Reyes-Ramirez *et al.*, 2003) and *Shewanella massilia* (Dos Santos *et al.*, 1998). Cells of strain HAW-EB4 were harvested from 266 ml aerobic culture (17 h), 820 ml nitrate-reducing culture (17 h), 410 ml TMAO-reducing culture (17 h) or 1600 ml culture incubated in the absence of any TEA (24 h). All subsequent anaerobic steps were carried out in an anaerobic glove box. Briefly, freshly prepared cell pellets were first rinsed once with 40 ml of an anaerobic buffer (10 mM Tris-HCl, 100 mM NaCl, pH 7.5) and then resuspended in an anaerobic sucrose-Tris buffer (10 mM Tris-HCl, pH 8.0, 0.5 M sucrose) with volumes ranging between 19 and 36 ml, giving cell densities ranging between 44 and 55 mg wet cells ml^{-1} . After equilibration at 21 °C for 7 min, the cell walls were digested with lysozyme (2 mg dissolved in 0.5 ml sucrose-Tris buffer) for 20 min. Subsequently, 0.2 ml EDTA (100 mM, pH 8.0) and an equal (to that of cell suspension) volume of Tris-HCl buffer (10 mM, pH 8.0) were added and incubated for another 15 min at room temperature to allow the release of periplasmic proteins by moderate osmotic shock and the complete formation of spheroplasts. By centrifugation (in tightened centrifugal bottles) at 27 000 g and 10 °C for 30 min, the periplasmic fraction of proteins was harvested as the supernatant (slightly pinkish) and spheroplasts as pellets. Periplasmic protein solution was filtered through a 0.45 µm pore-size membrane filter (Millex HV, PVDF) (Millipore) for further use. The spheroplasts were disintegrated by sonication in an anaerobic glove box (at a 70% output, ~22 W, setting 14, three 10 s intervals), followed by centrifugation (in tightened centrifugal bottles) at 17 000 g at 10 °C for 15 min to harvest cytoplasmic and membrane-bound proteins as the supernatant. The protein content was determined using the bicinchoninic acid method following the instructions of the BCA Protein Assay kit (Pierce). The protein content (mg ml^{-1}) in the periplasmic fraction prepared from cells grown on O₂ (air), nitrate or TMAO, or in the absence of any TEA, was 1.7, 1.4, 1.2 or 1.3, respectively. The isolated periplasmic proteins were kept at 4 °C in sealed serum bottles under N₂ (3% H₂, v/v).

In vitro anaerobic RDX metabolism by periplasmic proteins in the presence of NADH.

In an anaerobic glove box, 0.5 ml RDX stock solution (40 mg l^{-1} in 10 mM Tris-HCl buffer, pH 8.0) was mixed with 0.5 ml of the above filtered periplasmic proteins in 10 ml vials. RDX biotransformation was initiated by the addition of 10 µl NADH stock solution (75 mM NADH, 10 mM Tris, pH 8). The vial was then sealed and incubated statically (outside the glove box) at 10 °C. Two controls were prepared, one containing RDX and NADH and the second containing RDX and the periplasmic proteins. Another reaction containing RDX, NADH and periplasmic protein was incubated in foam-plugged vials to test aerobic RDX biotransformation. All tests were run in triplicate. The reaction was stopped at

3, 9 or 25 h by aeration. The whole vial was sacrificed for analysis of substrate RDX and products, as described below.

UV-visible spectroscopy of cytochromes. The cell suspension (OD₆₀₀ 0.9) and periplasmic protein solution as prepared above were placed in a quartz cuvette for UV-visible measurements. To oxidize cytochromes, cell suspension or periplasmic protein samples were aerated by gently pipetting samples three times. To reduce cytochromes, a few grains of sodium dithionite were added to the cell suspension and periplasmic protein solution and mixed for 2 min. The UV-visible spectra (300–700 nm) of the above samples were measured on a Beckman DU 640 UV-visible spectrophotometer (Beckman Instruments) (speed, 1200 nm min^{-1} ; interval, 25 s). Tris-HCl (pH 8, 10 mM) buffer was used as a blank for the periplasmic sample, whereas sea water was used as a blank for the whole-cell suspension. Reduced minus oxidized spectra were obtained by measuring spectra of the reduced sample using the oxidized sample as a blank.

Determination of cytochromes by SDS-PAGE analysis. SDS-PAGE analyses of periplasmic proteins were conducted on an XCell SureLock Mini-Cell using a gradient gel (NuPAGE Novex Bis-Tris gels, 4–12%, 0.1 × 8 × 8 cm) and a homogeneous buffer (NuPAGE MES SDS running buffer: MES, 2.5 mM; Tris base, 2.5 mM; SDS, 5%, w/v; pH 7.3) according to the instructions from Invitrogen. To detect haem-containing protein, periplasmic proteins were concentrated 17-fold (12-fold for those in aerobic cells) using a Microcon centrifugal filter device (Ultracel YM-10, Millipore). Fifteen microlitres of concentrated protein samples containing 140 µg proteins (160 µg protein for cells grown on nitrate) were loaded. The SeeBlue Plus2 pre-stained standard (Invitrogen) was also loaded (10 µl) as a molecular marker that contained myosin (188 kDa), phosphorylase (98 kDa), BSA (62 kDa), glutamic dehydrogenase (49 kDa), alcohol dehydrogenase (38 kDa), carbonic anhydrase (28 kDa), myoglobin red (17 kDa), lysozyme (14 kDa), apoferritin (6 kDa) and insulin B chain (3 kDa). The gel ran at 200 V for 45 min. To detect haem proteins, the gel was stained by incubation in a solution of 3,3',5,5'-tetramethylbenzidine (TMBZ) for 2 h, followed by the addition of 3 ml H₂O₂ (30%) (Thomas *et al.* 1976; Morris *et al.* 1990). Blue-coloured bands that indicated haem-containing proteins appeared after 10–60 min incubation in the dark at room temperature.

RDX oxidation of dithionite-reduced cytochrome. To prepare reduced cytochromes, 0.5 ml of periplasmic protein solution was incubated in a 1.5 ml Eppendorf tube briefly (2 min) with a few grains of sodium dithionite in an anaerobic glove box. The salts in the mixture were removed by ultrafiltration using a Microcon centrifugal filter device with an Ultracel YM-10 (molecular weight cut-off 10 kDa) membrane filter (Millipore) and a bench-top Sanyo Micro Centaur Microcentrifuge (MSB010 CX1.1; 11 600 g, 45 min) placed inside an anaerobic glove box. The protein retentate was rinsed with 0.3 ml Tris-HCl buffer (10 mM, pH 8.0) and further concentrated by centrifugation. The obtained dithionite-free protein retentate was then reconstituted with 0.5 ml anaerobic Tris buffer (10 mM, pH 8.0) and mixed with an equal volume of RDX solution (40 mg l^{-1} in 10 mM Tris-HCl buffer, pH 8) in a quartz cuvette. A control was prepared by mixing 0.5 ml of the above dithionite-free reduced cytochrome sample with an equal volume of Tris buffer (pH 8, 10 mM) in a quartz cuvette. Mineral oil (0.3 ml) was added to cover the media in cuvettes before incubation at 10 °C outside the glove box. The cuvettes containing the reaction medium and control were scanned periodically to measure their visible absorption spectra (400–700 nm) using Tris-HCl buffer (10 mM Tris-HCl, pH 8) as a blank. After the completion of the experiment, RDX remaining in the reaction medium and products was measured as described below.

Monitoring of cytochrome by spectrophotometry during in vitro incubation with RDX and NADH. In an anaerobic glove box,

0.5 ml of periplasmic protein solution was mixed in a quartz cuvette with an equal volume of RDX stock solution (40 mg l^{-1} , in Tris-HCl buffer) and $10 \mu\text{l}$ of 75 mM NADH stock solution (in Tris-HCl buffer). A control was prepared by mixing 0.5 ml of periplasmic protein solution with an equal volume of Tris-HCl buffer ($\text{pH } 8$, 10 mM) and $10 \mu\text{l}$ of 75 mM NADH stock solution (in 10 mM Tris-HCl buffer). Mineral oil was added to cover the reaction and control medium to maintain anaerobic conditions. All reactions and controls were then incubated statically at 10°C in an incubator outside the anaerobic glove box. During incubation, the cuvettes containing the reaction medium and control were scanned periodically to measure their UV-visible absorption spectra ($300\text{--}700 \text{ nm}$) using Tris buffer as a blank. NADH concentration was monitored by its absorbance at 340 nm . After the completion of the experiment, RDX remaining in the reaction medium and products was measured as described below.

Chemical analysis. The concentrations of RDX, MNX, DNX and TNX were determined by HPLC, as described previously (Hawari *et al.*, 2000). NDAB and MEDINA were determined on an AnionSep Ice-Ion-310 Fast organic acids HPLC column ($6.5 \times 150 \text{ mm}$; Cobert Associates Chromatography Products) at 225 nm and 35°C (Zhao *et al.*, 2004b). The methods for analysis of NO_2^- , N_2O and HCHO were as described in a previous report (Zhao *et al.*, 2002). All tests were performed in triplicate.

RESULTS AND DISCUSSION

Psychrophilic growth of *S. halifaxensis* in cold (10°C) seawater on various TEA

Strain HAW-EB4 grew rapidly under aerobic conditions in cold (10°C) seawater in the presence of peptone and yeast extract (Fig. 2a), with very poor growth observed under O_2 -limiting (data not shown) or fully anaerobic conditions in the absence of any external TEA (Fig. 2b). The addition of TMAO (Fig. 2c) significantly enhanced anaerobic growth of

strain HAW-EB4. In the presence of 10 mM TMAO and with an initial OD_{600} of 0.1 , biomass (OD_{600}) increased fivefold to 0.5 in 30 h . In the presence of 100 mM TMAO and with an initial OD_{600} of 0.5 , OD_{600} increased threefold to 1.5 in 30 h . Growth in the presence of TMAO was accompanied by an increase in pH to 9 due to the reduction of TMAO to alkaline trimethylamine.

The addition of nitrate also stimulated anaerobic growth of strain HAW-EB4 (Fig. 2d), but growth on nitrate was lower than that on TMAO. For example, in the presence of 20 mM nitrate and with an initial OD_{600} of 0.1 , biomass (OD_{600}) tripled to 0.3 in 30 h (Fig. 2d). In the presence of 50 mM nitrate and with an initial OD_{600} of 0.5 , OD_{600} only increased by 50% to 0.77 in 30 h (Fig. 2d). During growth on nitrate, N_2O accumulated, indicating incomplete denitrification. Therefore, nitrate reduction is not an energy-efficient respiratory process for the growth of strain HAW-EB4.

RDX did not increase the anaerobic growth of strain HAW-EB4 in MB 20 medium in comparison to the control that contained no RDX, suggesting that RDX did not serve as a TEA for the growth of this bacterium.

RDX removal kinetics in *S. halifaxensis* regulated by TEA

Strain HAW-EB4 degraded RDX when growing at 10°C in the presence of TMAO or under O_2 -limiting conditions, or being incubated in the absence of any TEA, but failed to degrade RDX under aerobic or nitrate-reducing conditions. These results showed that the presence of TMAO, O_2 -limitation and the absence of TEA favoured bacterial degradation of RDX in *S. halifaxensis* strain HAW-EB4.

Fig. 2. Biomass increase of *S. halifaxensis* HAW-EB4 (initial OD_{600} 0.1 or 0.5) during incubation at 10°C in MB 20 medium under aerobic (a) or anaerobic conditions (c–d), in the presence of TMAO (c) or nitrate (d), or in the absence of any TEA (b).

Fig. 3 shows the biotransformation of RDX by 4.1 ± 0.3 g wet weight l^{-1} (OD_{600} 3.4) of resting cells of strain HAW-EB4 obtained after 12.5 h of incubation (exponential phase) at 10 °C under TMAO-reducing conditions (Fig. 3a), in the absence of TEA (Fig. 3b), or under O_2 -limiting (not shown, but similar to Fig. 3b), nitrate-reducing (Fig. 3c) or aerobic conditions (Fig. 3d). Cells pre-incubated under anaerobic conditions in the presence (Fig. 3a) or absence (Fig. 3b) of TMAO, or under O_2 -limiting conditions (not shown, but similar to Fig. 3b), removed RDX at similar rates ($9.3\text{--}11\text{ }\mu\text{M h}^{-1}$), much faster than cells pre-grown on nitrate ($3.2\text{ }\mu\text{M h}^{-1}$) (Fig. 3c) or O_2 (air) ($0.36\text{ }\mu\text{M h}^{-1}$) (Fig. 3d). As reported in a previous study (Zhao *et al.*, 2004b), aerobically grown cells of strain HAW-EB4 degrade RDX in

anaerobic sea water suspension. The present results demonstrated that aerobically grown cells were about 10–30 times slower than anaerobically grown cells in removing RDX. These data further prove that TMAO-reducing and O_2 -limiting conditions, or the absence of TEA, enhance the removal of RDX by strain HAW-EB4. Of the three anaerobic conditions, TMAO-reducing conditions were optimal for both the growth of strain HAW-EB4 and RDX removal; therefore, the presence of TMAO would be expected to improve RDX removal in sediments in which strain HAW-EB4 is present.

Resting cells of strain HAW-EB4 obtained after anaerobic incubation (24 h) in the presence or absence of RDX in

Fig. 3. Anaerobic biotransformation of RDX (90 μM) at 10 °C in a sea water suspension (cell density close to OD_{600} 3.4, pH 7.5) of cells pre-grown in the presence of TMAO (a), nitrate (c) or O_2 (air) (d), or pre-incubated in the absence of any TEA (b).

MB 20 medium displayed similar RDX removal rates. Similar RDX removal rates were also found in cells pre-grown anaerobically in the presence or absence of RDX in MB 20 + TMAO medium. Both sets of experimental data showed that RDX did not further induce the enzymic activity for its removal in *S. halifaxensis* HAW-EB4. Instead, for TMAO-reducing conditions (Fig. 2c), cells harvested at mid (8 h) or late (14 h) exponential phase, or early (24 h) or late (48 h) stationary phase, displayed similar rates of RDX removal, demonstrating that RDX removal activity is constitutive and co-metabolic.

On the other hand, when resting cells of strain HAW-EB4 were incubated aerobically with RDX, none of the cells harvested under the previously described five conditions removed RDX, indicating that the enzyme(s) responsible for removal of RDX in this bacterium is O₂ sensitive. Because of O₂ and nitrate inhibition (as found in the assays that employed growing cells) of RDX metabolism, aerobically and nitrate-grown cells must first penetrate to the anoxic and nitrate-poor layer of marine sediment, where UXO are most likely buried, for RDX removal to occur.

RDX metabolic pathways in *S. halifaxensis* regulated by TEA

Previously, aerobically grown cells of *S. halifaxensis* HAW-EB4 were shown to degrade RDX to both nitroso (MNX, DNX and TNX; Fig. 1, path b) and ring cleavage (nitrite, MEDINA, HCHO and N₂O; Fig. 1, path a) products (Zhao *et al.*, 2004b). The present results showed that cells pre-grown anaerobically on TMAO or nitrate, or pre-incubated in the absence of any TEA, also degraded RDX via both the denitration route, giving the ring cleavage products (MEDINA, HCHO and N₂O) (Fig. 1, path a), and the nitroso route, giving the nitroso products (MNX, DNX and TNX) (Fig. 1, path b). However, the nitroso route (Fig. 1, path b) was predominant in cells grown on TMAO (Fig. 3a) or pre-incubated in the absence of TEA (or under O₂-limiting conditions) (Fig. 3b). For example, for TMAO-grown cells, MNX (5.7 $\mu\text{M h}^{-1}$) and DNX (1.5 $\mu\text{M h}^{-1}$) accounted for 70 % of initial RDX removal (9.3 $\mu\text{M h}^{-1}$). In the case of cells pre-incubated under anaerobic conditions in the absence of any TEA, MNX (8.5 $\mu\text{M h}^{-1}$) and DNX (0.73 $\mu\text{M h}^{-1}$) accounted for 82 % of initial RDX removal (11 $\mu\text{M h}^{-1}$ at 5 h). In comparison, MNX formed in aerobically (Fig. 3d) or nitrate-grown (Fig. 3c) cells accounted for 20–26 % of initial RDX removal. RDX transformation by TMAO-grown cells was accompanied by the formation of nitrite, MEDINA and HCHO, at respective rates of 0.73, 0.9 and 1.9 $\mu\text{M h}^{-1}$, clearly demonstrating ring cleavage following denitration (Fig. 1, path a) that accounted for 30 % of initial RDX removal. For cells incubated anaerobically in the absence of TEA, 18 % of initial RDX removal was found to be ring cleavage products, including nitrite (0.4 $\mu\text{M h}^{-1}$), MEDINA (0.85 $\mu\text{M h}^{-1}$) and HCHO (1.1 $\mu\text{M h}^{-1}$). However, for the less active nitrate (Fig. 3c) and aerobically (Fig. 3d) grown cells, up to 76 % of initial RDX removal was due to the formation of MEDINA

and HCHO, demonstrating the dominance of RDX denitration in cells grown under these two conditions. In all cases, NDAB, a product found in biodegradation of RDX by aerobic bacteria (Fournier *et al.*, 2002; Bhushan *et al.*, 2003) or hydrolysis of RDX or MNX (Balakrishnan *et al.*, 2003), was only detected as a minor anaerobic RDX product with a formation rate 5–10 times lower than that of MEDINA (Fig. 3).

These results showed that RDX in marine sediment under all five possible field growth conditions should degrade to both nitroso and ring cleavage products. Better removal and more intermediary formation of nitroso derivatives should be expected if TMAO is present in anoxic environments. It should be noted that in the assays that used growing cells, none of the nitroso derivatives, including TNX, was found to persist indefinitely; rather, they disappeared to produce HCHO, CO₂ and N₂O.

In vitro anaerobic RDX metabolism by periplasmic proteins

To determine the cellular location of proteins that metabolized RDX, subcellular fractions including spheroplast and periplasmic fractions of cells of strain HAW-EB4 were prepared using a moderate osmotic shock-based method (Birdsell & Cota-Robles, 1967; Easter *et al.*, 1983) to test their potential to metabolize RDX. The proteins in the periplasmic fraction of TMAO-grown cells showed NADH-dependent anaerobic RDX degradation activity (within 5 h), whereas no RDX removal was found in cytoplasmic and membrane-bound proteins or in the controls that contained NADH or periplasmic proteins alone. Similarly, RDX metabolic activity was found in periplasmic proteins of cells grown in the presence of nitrate or O₂ (air), or pre-incubated in the absence of any TEA. These data indicate that the proteins responsible for RDX metabolism are located in the periplasmic space of strain HAW-EB4.

Likewise, periplasmic proteins from cells grown on TMAO (14 $\mu\text{M h}^{-1}$; Fig. 4a) or pre-incubated in the absence of any TEA (21 $\mu\text{M h}^{-1}$; Fig. 4b) transformed RDX at rates faster than those of periplasmic proteins from nitrate- (8.3 $\mu\text{M h}^{-1}$; Fig. 4c) or aerobically (2.3 $\mu\text{M h}^{-1}$; Fig. 4d) grown cells (in all cases, protein content was 0.6–0.85 mg ml⁻¹). As for the distribution of products, periplasmic proteins of cells grown on TMAO (Fig. 4a) or pre-incubated in the absence of any TEA (Fig. 4b) gave nitrite, MEDINA and HCHO as ring cleavage products (Fig. 1, path a), and MNX, DNX and TNX as nitroso products (Fig. 1, path b). As found in whole-cell assays, periplasmic proteins of nitrate (Fig. 4c) or aerobically (Fig. 4d) grown cells metabolized RDX mainly via cleavage to MEDINA and HCHO (Fig. 1, path a). No NDAB was detected in any of the above anaerobic bio-transformations of RDX with periplasmic proteins.

Periplasmic proteins obtained from cells grown on TMAO, nitrate or O₂ (air), or pre-incubated in the absence of any TEA, were unable to metabolize RDX under aerobic

Fig. 4. Anaerobic biotransformation of RDX (89 µM) in the presence of NADH (0.75 mM) by periplasmic proteins from cells pre-grown in the presence of TMAO (a), nitrate (c) or O₂ (air) (d), or pre-incubated in the absence of any TEA (b). Reaction volumes: (a), 1 ml; (b–d), 2 ml. The protein contents for (a), (b), (c) and (d) were 0.6, 0.65, 0.7 and 0.85 mg ml⁻¹, respectively.

conditions, consistent with the O₂ inhibition of RDX metabolism found in whole-cell assays.

Regulation by TEA of *c*-type cytochrome production in *S. halifaxensis*

Several strains of *Shewanella* have been reported to produce *c*-type cytochromes capable of reducing insoluble metal oxide (Tsapin *et al.*, 1996, 2001; Gordon *et al.*, 2000). In the present study, the cytochrome content of *S. halifaxensis* HAW-EB4 grown under both anaerobic and aerobic conditions was characterized and determined for its potential involvement in RDX metabolism. Table 1 shows that freshly prepared or dithionite-reduced periplasmic proteins from cells grown on TMAO or pre-incubated in the absence of TEA had maximal absorption bands at 418–419, 522–523 and 551 nm. Similar bands (417–418, 521–522 and 551 nm) were also found in dithionite-reduced periplasmic proteins from cells grown on nitrate and O₂ (air) (Table 1). The absorption maxima at 417–419, 522 and 551 nm corresponded, respectively, to the γ (Soret), β and α bands of *c*-type cytochrome in the reduced state (Poole, 1994), and are similar to those of *c*-type cytochromes found in other strains of *Shewanella* (Tsapin *et al.*, 2001; Pitts *et al.*, 2003; Yamada *et al.*, 2000). Freshly prepared or air-oxidized periplasmic proteins from nitrate- or O₂ (air)-grown cells showed a single maximal band at 410 or 413 nm, corresponding to the γ band of oxidized *c*-type cytochrome. A similar γ band of *c*-type cytochrome was found at 408–409 nm in oxidized (after

brief exposure to air) periplasmic proteins from cells grown on TMAO or pre-incubated in the absence of TEA. Furthermore, the reduced minus oxidized difference spectra of all periplasmic proteins and whole-cell suspensions (data not shown) also clearly showed the γ (419–420 nm), β (522 nm) and α (551 nm) bands of *c*-type cytochrome (Table 1), demonstrating the production of *c*-type cytochromes by strain HAW-EB4 grown under all four tested conditions.

The cytochromes prepared from cells harvested under the four incubation conditions displayed differences in their UV–visible spectra (Table 1). For example, the wavelength of the γ band (409 nm) of air-oxidized cytochrome of TMAO-grown cells was lower than that of aerobically (413 nm) or nitrate (410 nm)-grown cells (Table 1). This suggests that strain HAW-EB4 produces different sets of *c*-type cytochromes in the presence of various TEA.

Using SDS-PAGE and subsequent staining with TMBZ, proteins containing covalently bound haem groups were detected in periplasmic proteins grown under all four conditions (Fig. 5). Because the haem group of the detected proteins could not be dissociated from protein by SDS, the haem group(s) must be covalently bound to the proteins, consistent with the structure of *c*-type cytochromes (Bartsch, 1968). Fig. 5 shows that periplasmic proteins of TMAO-grown cells had about six haem protein bands, ranging from 14 to 80 kDa, and that the band larger than 62 kDa was the predominant one. In contrast, periplasmic proteins of nitrate-grown cells had two smaller haem

Table 1. Maximal absorption peaks of *c*-type cytochrome detected in periplasmic proteins of *S. halifaxensis* grown in the presence of various TEA

+, Peak detected; –, peak not detected; R–O, spectra of reduced sample (R) minus spectra of air-oxidized sample (O). The wavelengths of maximal peaks that differed from the value shown in the peaks column are in parentheses.

TEA used	Peaks (nm)	Peaks in original proteins	Relative heights of peaks of cytochromes detected in total periplasmic proteins*				Matched peaks of <i>c</i> -type cytochrome, redox state
			Air-oxidized (O)†	Dithionite-reduced (R)‡	NADH-reduced‡	Difference spectra (R–O)†	
TMAO	409	–	37	–	–	–	γ , oxidized
	419	+	–	59	19 (418)	63(420)	γ , reduced
	522	+	–	5	2	3	β , reduced
	551	+	–	8	3.2	8.6	α , reduced
None	408	–	36	–	–	–	γ , oxidized
	418	+	–	53	14	60 (420)	γ , reduced
	522	+	–	5 (523)	1.7	3	β , reduced
	551	+	–	7.6	2.6	8.5	α , reduced
Nitrate	410	+	18 (409)	–	–	–	γ , oxidized
	417	–	–	21	4.7	24 (419)	γ , reduced
	522	–	–	1.7	0.6	1	β , reduced
	551	–	–	3.1	1.1	3.2	α , reduced
O ₂ (air)	413	+	14	–	–	–	γ , oxidized
	418	–	–	19	2.7 (417)	16 (419)	γ , reduced
	522	–	–	1.6	0.03 (521)	0.7	β , reduced
	551	–	–	2.5	–	2.1	α , reduced

*Relative peak height was the difference between absorbance at the peak and at the base, multiplied by 100. The respective bases for the peaks at 409, 418, 520 and 551 nm were at 400, 438, 500 and 500 nm.

†Periplasmic protein content was 1.2–1.7 mg protein ml^{–1}.

‡Periplasmic protein content was 0.6–0.85 mg protein ml^{–1}.

proteins ranging from 14 to 28 kDa, whereas those of cells pre-incubated in the absence of TEA had a band close to (but smaller than) 62 kDa. In the case of aerobically grown cells, the cytochrome content was so low that one haem protein between 14 and 28 kDa was only detected in high protein-loading (450 µg; three times the 140 µg for TMAO-grown cells) SDS-PAGE analysis, in which protein separation was rather poor (data not shown). These results demonstrated that *S. halifaxensis* HAW-EB4 grown on various TEA had different sets of *c*-type cytochromes, and produced several more *c*-type cytochromes in the presence of TMAO.

On the other hand, crude RDX metabolic proteins salted out from periplasmic proteins of TMAO-grown cells using 50–65 % (NH₄)₂SO₄ saturation were found to contain highly concentrated *c*-type cytochromes (spectra shown in Fig. 6a). The crude cytochrome preparation also catalysed an NADH-dependent RDX biotransformation to products (Fig. 6b) similar to those found in the case of total periplasmic proteins (Fig. 4c). In both cases, the nitroso derivatives resulting from stepwise reduction of -N-NO₂ to -N-NO, and the ring cleavage products (MEDINA, HCHO, N₂O) resulting from initial denitration were detected. This

experimental observation further suggests that *c*-type cytochromes are linked to RDX metabolism.

Evidence for involvement of *c*-type cytochromes in RDX metabolism

To provide further evidence for the involvement of *c*-type cytochromes in RDX metabolism, we obtained crude reduced cytochromes by using dithionite to reduce each type of periplasmic protein, followed by ultrafiltration to remove salts. When the reduced crude cytochromes were anaerobically incubated with RDX, they were rapidly oxidized, as indicated by the faster decrease in absorbance at the γ (418–420 nm) and α (551 nm) bands of reduced cytochrome (as shown in Fig. 7a1 for cytochrome in TMAO-grown cell) compared to that of the controls containing no RDX (Fig. 7a2). Table 2 shows that dithionite-reduced cytochromes prepared from cells grown on TMAO or pre-incubated in the absence of TEA were oxidized at rates (as indicated by the rate of decrease in absorbance of the γ or α band) that were much faster (four to eight times faster by the γ band; two to five times faster by the α band) in the presence of RDX than in the absence of RDX. Dithionite-reduced cytochromes from nitrate or

Fig. 5. Detection of *c*-type cytochromes by SDS-PAGE (4–12 %, w/v, gradient gel) analysis and haem staining. Protein loading was 140–160 μ g. Haem staining was conducted by stepwise incubation of the gel with TMBZ and H_2O_2 , as described elsewhere (Thomas *et al.*, 1976). Periplasmic proteins from cells incubated under aerobic, nitrate-reducing and TMAO-reducing conditions, and in the absence of TEA are in lanes 2, 3, 4 and 1, respectively. Lane M, marker proteins; approximate sizes are indicated in kDa on the right-hand side.

aerobically grown cells were only oxidized (as indicated by the decrease in absorbance at the γ or α band) in the presence of RDX, with little oxidation in the controls containing no RDX (Table 2). Cytochromes from cells grown on TMAO or from cells pre-incubated in the absence of TEA also appeared to be more easily (10–30 times faster by the rate of decrease in absorbance of the γ or α band) oxidized by RDX than cytochrome from nitrate- or aerobically grown cells (Table 2), consistent with the trend of rates for RDX removal found earlier in assays using periplasmic proteins (Fig. 4).

During *in vitro* anaerobic incubation of periplasmic proteins from all four types of cells with NADH and in the absence of RDX, cytochromes were rapidly reduced, as indicated by the appearance of a sharp γ band at 417–418 nm and an α band at 551 nm (Table 1, Fig. 7b2). Cytochrome reduction was also observed during *in vitro* anaerobic incubation of periplasmic proteins with both NADH and RDX, especially in the early stage (Fig. 7b1); however, after 150 min incubation, the absorbance of the γ or α band of reduced cytochrome in the presence of RDX was approximately two to ten times lower than that in the controls containing no RDX (Table 2). Fig. 7(b1) clearly illustrates a rapid oxidation of NADH-reduced cytochrome from TMAO-grown cells by RDX compared to that in the RDX-free control, in which cytochrome remained reduced under similar conditions (Fig. 7b2). These data demonstrated that RDX oxidized reduced cytochromes during anaerobic RDX biotransformation by periplasmic proteins in the presence of NADH. Consistent with these findings, NADH oxidation rates (as measured by the rate of decrease in absorbance at 340 nm; Table 2) during RDX biotransformation were seven to 38 times faster than those found in controls that contained no RDX. The faster NADH oxidation during RDX biotransformation was attributed to *c*-type cytochrome, since RDX alone did not oxidize NADH in the abiotic control without periplasmic protein (cytochrome) (data not shown). These findings, together with those observed in tests with dithionite-reduced cytochromes, clearly demonstrate that *S. halifaxensis* HAW-EB4 employs *c*-type cytochromes in RDX metabolism.

As mentioned above (Table 1), the reduced cytochrome in each type of cell was very sensitive to O_2 and could be instantly oxidized by a brief exposure to air. This is in line with RDX persistence when incubated with each type of periplasmic protein and NADH under aerobic conditions.

On the other hand, auto-oxidation of dithionite-reduced cytochromes in cells grown on TMAO or pre-incubated in the absence of TEA was much faster than that of reduced

Fig. 6. Crude cytochromes from periplasmic proteins in TMAO-grown cells [salted out with 50–65 % $(NH_4)_2SO_4$ saturation]. (a) Absorption spectra (2.3 mg protein ml^{-1}); (b) RDX biotransformation products in the presence of NADH (4.7 mg protein ml^{-1}).

Fig. 7. Visible spectra of (a) dithionite- or (b) NADH-reduced *c*-type cytochromes from TMAO-grown cells during anaerobic incubation in the presence (a1, b1) or absence of RDX (a2, b2).

cytochrome from nitrate- or aerobically grown cells, as shown in Table 2, suggesting that cytochromes in the former two types of cells have a lower redox potential.

All the above data demonstrate that strain HAW-EB4 incubated with various TEA produces different sets of *c*-type cytochromes for co-metabolism of RDX. The present

findings are believed to provide the first evidence for involvement of *c*-type cytochrome in anaerobic RDX metabolism. *c*-Type cytochromes have been reported for their enzymic activity to reduce sulfur (Fauque *et al.*, 1979), U (VI) (Lovley *et al.*, 1993), Fe (III) (Aubert *et al.*, 1998), Cr (IV) (Lovley & Phillips, 1994) and nitrite (Pooch *et al.*, 2002; Liu & Peck, 1981; Einsle *et al.*, 2000). Other

Table 2. RDX oxidation of dithionite- or NADH- reduced *c*-type cytochrome prepared from cells grown on various TEA

ND, No data.

TEA for growth of cells as cytochrome sources	Total protein concn in reaction medium (mg ml ⁻¹)	Relative rate for oxidation of cytochrome reduced by dithionite*				Relative absorbance (<i>A</i>) of reduced cytochrome remaining after 150 min incubation with NADH†				Relative rate for oxidation of NADH in the presence of cytochrome‡ (rate by <i>A</i> ₃₄₀)	
		Rate by <i>A</i> _γ		Rate by <i>A</i> _α		<i>A</i> _γ		<i>A</i> _α		+RDX	-RDX
		+RDX	-RDX	+RDX	-RDX	+RDX	-RDX	+RDX	-RDX		
TMAO	0.6	54	7	10	2	4	18	1	3	30	2
None	0.65	95	22	10	5	4	15	1	3	38	1
Nitrate	0.7	4	0.03	1	0.01	3	6	0.9	1.4	21	3
O ₂ (air)	0.85	3	0	0.3	0	<0	2.2	0.06	0.56	ND	ND

*The relative rate of oxidation of reduced cytochrome is equal to the rate of decrease (per minute) in absorbance at either the γ (*A*_γ) or the α (*A*_α) band multiplied by 10⁴ (during 370 min anaerobic incubation in cuvettes covered with mineral oil).

†The relative *A*_γ or *A*_α of reduced cytochrome is equal to the difference between absorbance at the peak (γ band at 417–420 nm; α band at 551 nm) and the base (400 nm for the γ band; 538 nm for the α band) multiplied by 10².

‡The relative rate is equal to the rate of decrease (per minute) in absorbance at 340 nm multiplied by 10⁴.

cytochromes, such as a eukaryotic cytochrome P450 from rabbit liver (Bhushan *et al.*, 2003) and a cytochrome P450-like protein (XplA) in the Gram-positive aerobic actinomycete *Rhodococcus rhodochrous* strain 11Y (Seth-Smith *et al.*, 2002), have been reported to be involved in RDX degradation. Both cytochrome P450 and XplA in *Rhodococcus* catalyse aerobic degradation of RDX, which is different from the enzyme of strain HAW-EB4 in the present study, which catalysed anaerobic RDX transformation alone. A preliminary search of genomes of *Shewanella* (in GenBank) by BLAST showed that the cytochrome P-450 (GenBank accession no. AAA31210) and XplA (GenBank accession no. AF449421) proteins were missing from the genomes of all sequenced strains of *Shewanella*, including the shotgun sequences of strain HAW-EB4 (J.-S. Zhao and others, unpublished data), suggesting that the present strain does not use XplA for RDX degradation.

c-Type cytochrome is known to mediate a $1e^-$ transfer process (Bartsch, 1968). In our case, a $1e^-$ nitro group reduction would lead to formation of an unstable anion free radical, which upon denitration would lead to ring cleavage and formation of MEDINA (Fig. 1, path a), consistent with those proposed in RDX metabolism by diaphorase (Bhushan *et al.*, 2002; Fig. 1). In an earlier study, an oxygen-sensitive reductase present in *Escherichia coli* and rat hepatic microsomes was found to reduce nitrofurazone to its nitroso and amine derivatives via stepwise $1e^-$ transfers (Peterson *et al.*, 1979). A similar mechanism might be used by the cytochromes in strain HAW-EB4 for the stepwise reduction of RDX to MNX, DNX and TNX (Fig. 1, path b).

In summary, the present experimental evidence shows that *S. halifaxensis* has a TEA-regulated co-metabolic RDX degradation activity that involves *c*-type cytochrome. *Shewanella* are known to produce diverse soluble low-redox-potential multihaem *c*-type cytochromes (Tsapin *et al.*, 1996, 2001; Gordon *et al.*, 2000), and genomes of multiple strains of *Shewanella* have been sequenced (<http://img.jgi.doe.gov/>). *Shewanella* is thus an ideal model organism to investigate the molecular mechanisms that underlie the TEA-regulated multiple electron-transfer pathways of anaerobic RDX biotransformation via either initial denitration or initial reduction of -N-NO₂ to -N-NO. In addition to other approaches, such as the recently described microbial electron shuttling (Bhushan *et al.*, 2006; Kwon & Finneran, 2006), the involvement of *c*-type cytochrome in anaerobic RDX reduction provides a basis for the development of a new strategy to improve RDX bioremediation in the anoxic environment.

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