CHARACTERIZATION OF CLOSTRIDIUM BIFERMENTANS AND ITS
BIOTRANSFORMATION OF 2,4,6-TRINITROTOLUENE (TNT) AND 1,3,5-TRIAZA-
1,3,5-TRINITROCYCLOHEXANE (RDX)

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SUMMARY

We have determined that an organism able to degrade both RDX and TNT in a pure culture is a strain of Clostridium bifermentans. The consortium from which this organism is derived also degrades these compounds, and we suspect that C. bifermentans is also the responsible organism within that consortium. The bioconversion of RDX and TNT occurs under anaerobic conditions both in the consortium and in pure culture without the need of an added reductant. The presence of co-metabolites speeded these biotransformations.

INTRODUCTION

Bacteria in the genus Clostridium have long been known for their ability to carry out novel bioconversions of unusual substrates. The products of these bioconversions are as varied as the substrates themselves. We have confirmed this metabolic versatility by isolating clostridia from an anaerobic digester fed munitions compounds as its sole source of carbon and energy (Funk et al., 1993). One isolate, a strain of Clostridium bifermentans, was able to transform the primary components of explosives, and was also able reproduce the sequence of events previously observed in the digester. That sequence was characterized by an initial reduction in the concentration of 2,4,6-trinitrotoluene (TNT) followed by concurrent reductions in the concentrations of both TNT and 1,3,5-triaza 1,3,5-trinitrocylohexane (RDX).

MATERIALS AND METHODS

To isolate the bacterium, 1 ml of our anaerobic consortium was used to inoculate 100 ml of anoxically prepared, sterile brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) supplemented with ~50 ppm RDX. The inoculated enrichment was reduced with dithionite at a final concentration of 0.001% and incubated at 35°C overnight. The enrichment flask was streaked for isolation of pure cultures on BHI broth solidified with Bacto agar (Difco) to a final concentration of 2%. BHI agar plates used for the isolation of potential TNT/RDX degraders contained no RDX. They were incubated at 35°C overnight in an anaerobic glove box. Individual colonies showing different macroscopic morphologies were then streaked to anoxically prepared
BHI slants containing 50 ppm of RDX. The inoculated slants were incubated 24 hours at 35°C to obtain good growth and sporulation of any sporogenic cultures, and stored at 4°C until analyzed.

Morphological types were tested alone and in mixtures for their ability to biotransform RDX in BHI. Of the isolates and mixtures tested, one isolate, a sporulating bacillus, removed RDX from the media more rapidly than the other isolates and mixtures (data not shown). This isolate was then tested for its ability, as compared to that of the batch fed anaerobic consortium from which it was isolated, to simultaneously transform TNT and RDX. These experiments were carried out in anaerobically prepared BHI media containing both TNT (~30 ppm) and RDX (~50 ppm). Inoculated flasks were prepared under one of three reducing conditions: non-reduced, cysteine-reduced, and dithionite-reduced. An isolate designated KMR-1, which showed the highest calculated percentage change in RDX after 72 hours, was physiologically characterized and identified by the AN-IDENT System (API Systems).

The chromosomal DNA was obtained from the clostridial isolate and its 16s ribosomal sequence amplified by PCR. The 16s amplified sequence was purified and cloned into pT7Blue T-vector (Novagen). This construct was introduced into NovabluE competent cells (Novagen) and several colonies containing the insert were selected. Three isolates were grown in a rich, selective medium for isolation of the T7Blue plasmid containing the insert. The plasmid DNA was isolated and prepared for dideoxysequencing using M13-Forward and M13-Reverse primers labeled with an infrared fluorophore. A Sequitherm and the Sequitherm cycle sequencing protocol (Epicenter Technologies Corp.) were used for direct sequencing. The sequencing gel was analyzed via the Li-Cor DNA 4000 (Li-Cor. Inc.) from three separate sequencing experiments with three isolated colonies.

To characterize the antibiotic sensitivities of the clostridial isolate KMR-1, it was tested against batteries of both traditional and non-traditional anti-clostridial antibiotics, using methods as described by Sutter (1985). The growth of strain KMR-1 was monitored by measuring the optical density (OD) at 600 nm in the presence of explosives and different reducing agents.

RDX and TNT concentrations were determined by reverse phase HPLC according to EPA Method 8330. Analysis was based on the solute elution times and spectra analysis, as compared to authentic standards run under identical conditions. An Ultracarb 5 ODS(20) 250 x 4.6 mm column (Phenomenex) was used for analysis. The solutes were eluted from the column by an isocratic mobile phase of 55% (v/v) methanol and 45% (v/v) water, at a flow rate of 0.5 ml/min. TNT and RDX, synthesized in our laboratory, were >99% pure (S. Goszczynski, pers. comm.).

To obtain scanning electron micrographs, cells were fixed with glutaraldehyde, progressively dehydrated with ethanol and fixed on aluminum carriers with carbon tape. Cells were sputter-coated with gold prior to observation with a Humer III (Techics). The preparations were examined using an AMRAY scanning electron microscope at 15.0 kV.

RESULTS AND DISCUSSION

Three morphological types were found among the isolates. Flat, translucent colonies with an entire edge were formed by strain KMR-1, a rod-shaped, gram-positive, obligately anaerobic strain that was motile, catalase-negative, and endospore-forming. It transformed TNT and RDX in BHI medium efficiently, and was chosen for further study. Physiological characterization by the API AN-IDENT system tentatively identified KMR-1 as a strain of Clostridium bifermentans. The four positive reactions obtained were indole production, leucine aminopeptidase, proline aminopeptidase, and motility; all others were negative. Using three colony isolates, the 16s ribosomal summarized sequence (Figure 1) containing 1024 bases was determined.