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14. ABSTRACT
2,4,6-trinitrotoluene (TNT) is the most widely used nitroaromatic compound, which is recalcitrant to degradation and has presented an environmental issue at many sites.
Among different microorganisms that act in TNT biodegradation, clostridium species were distinguished for their rapid degradation rate. Here we compared different C. acetobutylicum strains for degradation of TNT in vivo. A series of mutant strains derived from C. acetobutylicum

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## Report Title

Final Report on Genetic Analysis of Nitroaromatic Degradation by Clostridium

## ABSTRACT

2,4,6-trinitrotoluene (TNT) is the most widely used nitroaromatic compound, which is recalcitrant to degradation and has presented an environmental issue at many sites. Among different microorganisms that act in TNT biodegradation, clostridium species were distinguished for their rapid degradation rate. Here we compared different C. acetobutylicum strains for degradation of TNT in vivo. A series of mutant strains derived from C. acetobutylicum ATCC 824, were tested for TNT degradation and only minor differences were observed. These studies showed that TNT was an effective competitor for reductant in cells and that TNT reduction altered the metabolic pattern of the culture. A number of clostridial strains were obtained and some tested for TNT degradation during growth on different carbon sources. Samples for microarray analysis were also obtained from various strains during the TNT degradation process. Analysis of the data from microarray experiments performed by the laboratory of C. Sund showed several genes related to redox were affected. Other molecules with TNT-like redox potentials were tested for their effects on metabolite pattern. Phenazine, a molecule produced by some soil bacteria was found to have a significant effect on metabolite pattern in two clostridium test strains and similar to that found with TNT.

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## Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

### (a) Papers published in peer-reviewed journals (N/A for none)

| Received | | Paper |
|---|---|---|
| 08/24/2012 | 1.00 | Matthew Servinsky, Xianpeng Cai, James Kiel, Christian Sund, George N. Bennett. Analysis of redox responses during TNT transformation by Clostridium acetobutylicum ATCC 824 and mutants exhibiting altered metabolism, Applied Microbiology and Biotechnology, (07 2012): 0. doi: 10.1007/s00253-012-4253-3 |

**TOTAL:** 1

**Number of Papers published in peer-reviewed journals:**

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### (b) Papers published in non-peer-reviewed journals (N/A for none)

| Received | Paper |
|---|---|

**TOTAL:**

**Number of Papers published in non peer-reviewed journals:**

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### (c) Presentations

George Bennett “Analysis of Redox Responses During TNT Transformation by Clostridium acetobutylicum ATCC824” Clostridium XII International Conference on the Genetics, Physiology and Biotechnology of Solvent- and Acid-forming Clostridia, September 10-12, 2012 Nottingham, UK

**Number of Presentations:** 1.00

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## Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

**TOTAL:**

**Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):**

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## Peer-Reviewed Conference Proceeding publications (other than abstracts):

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**Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):**

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## (d) Manuscripts

Received Paper

**TOTAL:**

**Number of Manuscripts:**

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## Books

Received Paper

**TOTAL:**

## Patents Submitted

Patent filed Rice: 2012-032USP WO 2013/096665 PCT/US2012/071038
TITLE: Long Chain Organic Acid Bioproduction INVENTORS: George N. BENNETT; Xianpeng CAI

## Patents Awarded

## Awards

George Bennett Waksman award from Society for Industrial Microbiology 2010
George Bennett elected as Fellow of American Association for the Advancement of Science 2011

## Graduate Students

| NAME | PERCENT_SUPPORTED |
| --- | --- |
| **FTE Equivalent:** | |
| **Total Number:** | |

## Names of Post Doctorates

| NAME | PERCENT_SUPPORTED |
| --- | --- |
| Chandresh Thakker | 0.10 |
| **FTE Equivalent:** | **0.10** |
| **Total Number:** | **1** |

## Names of Faculty Supported

| NAME | PERCENT_SUPPORTED | National Academy Member |
| --- | --- | --- |
| George Bennett | 0.00 | |
| **FTE Equivalent:** | **0.00** | |
| **Total Number:** | **1** | |

## Names of Under Graduate students supported

| NAME | PERCENT_SUPPORTED | Discipline |
| --- | --- | --- |
| Crystal Boafo | 0.00 | Biochemistry & Cell Biology |
| Juyeon Hong | 0.00 | Biochemistry & Cell Biology |
| Austin Potter | 0.00 | Biochemistry & Cell Biology |
| Emily Adkins | 0.00 | Chemical Engineering |
| David French | 0.00 | Biochemistry & Cell Biology |
| **FTE Equivalent:** | **0.00** | |
| **Total Number:** | **5** | |

**Student Metrics**

This section only applies to graduating undergraduates supported by this agreement in this reporting period

| | |
|---|---|
| The number of undergraduates funded by this agreement who graduated during this period: ...... | 5.00 |
| The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:...... | 5.00 |
| The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:...... | 3.00 |
| Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale): ...... | 4.00 |
| Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering: ...... | 0.00 |
| The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense ...... | 0.00 |
| The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: ...... | 1.00 |

## Names of Personnel receiving masters degrees

NAME

**Total Number:**

## Names of personnel receiving PHDs

NAME

**Total Number:**

## Names of other research staff

| NAME | PERCENT_SUPPORTED |
|---|---|
| Mary Harrison | 0.30 |
| **FTE Equivalent:** | **0.30** |
| **Total Number:** | **1** |

## Sub Contractors (DD882)

## Inventions (DD882)

# Scientific Progress

2. Scientific Progress and Accomplishments

## 2.1 Mutations affecting solvent and acid production do not affect TNT degradation rate significantly in several genetically modified strains

In our laboratory, several genetic modified strains have been constructed for studies of solvent production. 824 (buk, butyrate kinase) and 824 (pta, phosphotransacetylase) are integrative recombinant mutants targeted for abolishing acid production. Mutant B is an integrative recombinant mutant designed for disruption of SolR a gene that affects solvent production. All three mutant strains displayed a different metabolite profile of acids and solvents from the wild type parent, Clostridium acetobutylicum 824-Mutant M5 is a degenerated strain which doesn't carry the mega plasmid bearing many solvent formation genes and consequently doesn't form solvent.
Samples from each culture were diluted for the TNT degradation rate assay at different time points, such as A600 0.3 (for early exponential phase), A600 1.0 (for mid-exponential phase) and 24-hour culture (for stationary phase culture, solventgenic phase). It was found that all strains tested showed similar TNT degradation rate, ranging from 10,000-12,000 μM/Hour/A600 during early exponential phase. All strains tested showed the same trend of TNT degradation rate decrease along with time. These results suggest that hydrogenase, which is more active in the early phase of the culture, is involved in the TNT degradation process and further indicate that hydrogenase is present in high amounts in all strains tested. The maximal TNT degradation rate may be limited by hydrogenase or by other factors such as the glucose uptake rate, growth rate, as well as other components in the media.

## 2.2 Analysis of the efficiency of TNT reduction vs glucose utilization

Glucose uptake rates for experiments were found by analyzing the amount of acetate and butyrate produced by Clostridia cultures (A=0.3) within an hour of TNT addition. Since the stoichiometric ratio of moles of metabolite produced to glucose consumed is 2:1 for acetate and 1:1 for butyrate, the molar increase observed in these two products during the first hour was used to estimate glucose metabolism. Other metabolites produced were negligible. Experiments analyzed a pair of identical TNT assays and the glucose uptake by the Clostridia culture (A=0.3) was analyzed by HPLC. For each experiment, the glucose concentration values were measured both before and after the hour-long run with TNT. The rate of glucose consumption was directly determined from the concentration difference. The rates of glucose uptake from outside literature were also obtained and used in the comparison, those were conducted in the absence of TNT. In each of these papers, information from profiles of glucose uptake by C. acetobutylicum cultures (A=1.0) was extrapolated to estimate the consumption rate in the conditions used in our own experiments. The ratios of the TNT transformation rate to glucose metabolism in our TNT assays ranged from around 0.5 to 1, which is comparable to the range extrapolated from the literature data using glucose uptake rates, 0.43-0.51. In all of the experiments, a mole of TNT was reduced for about every 1-2 moles of metabolized glucose. These relatively high ratios suggest that TNT can be reduced competitively against regular cellular metabolism. This suggests the TNT can be an effective sink for electrons and competes well with native hydrogen producing reactions in the cell when we examine the culture at the maximal rate of TNT reaction. Thus rather than being a minor side reaction of the cells metabolism the TNT is an effective in vivo reactant with the redox carriers/enzymes such as hydrogenase, and it can affect the cells metabolism during this time. The reaction with TNT not only can use a significant proportion of the cells reducing power, but in doing this it affects the overall pattern of metabolism and is in turn affected by competitors which influence the redox situation, e.g. the more effective diversion of redox to solvent producing redox pathways later in the culture. This perspective would place TNT among the group of dyes that are metabolized by clostridia, and thus this work could have broader implications in the general control/understanding of redox regulation and response in these anaerobes. In the case of TNT the reduction is irreversible and continues beyond an initial step while for many dyes the reaction is reversible and the reduced dye can transfer electrons to another carrier or redox reaction and affect the metabolite pattern as noticed with the well-known observation that neutral red and violgen addition results in higher butanol levels.

## 2.3 The metabolite pattern is affected during TNT reduction

In experiments by C. Sund, he found that when TNT was added the hydrogen production desreased. This would be expected if TNT is acting at the hydrogenase and taking some of the electrons that would normally go to hydrogen formation to instead reduce the TNT. We measured the acid metabolites formed during the time after TNT addition. A culture grown under the conditions where the TNT degradation rate was analyzed was processed and subjected to gas chromatography to determine the metabolites present in the pre-TNT added culture and in the culture 1 hour after the TNT addition, a time when the starting TNT had been depleted. The culture continued to grow during this time and the metabolism of glucose continued during the 1 hour period and seemed unchanged in rate as judged by the production of acids during this time. Analysis of the metabolites formed over the period when TNT is being transformed shows a significant change in the proportions of acetate and butyrate, with a large amount of butyrate eing produced in comparison to very little acetate.
If one considers the total redox available and that some of the redox goes to reduce the TNT, initially it might seem that the loss of this reducing power would lessen the amount of butyrate formed, since formation of butyrate from pyruvate requires more reduction than formation of acetate. However if we examine the process of reduction to form hydrogen and butyrate we see a

difference in cofactor used. In the formation of hydrogen, the main source is reduced ferredoxin generated by the pyruvate:ferredoxin oxidoreductase in the conversion of pyruvate to acetyl-CoA, and this is supplemented to some degree by conversion of some of the NADH formed in glycolysis via a NADH-ferredoxin reductase. In the early growth stage we are studying there is no formation of the reduced solvents, ethanol and butanol and the enzymes forming these compounds are not induced under this growth condition. In this circumstance the shift to more butyrate formation during TNT reduction by the ferredoxin-Fe hydrogenase can be considered as a result of blockage of the usual hydrogenase acceptance of reductant from reduced ferredoxin, and thus the backing-up of the NADH-ferredoxin reductase reaction and its contribution to hydrogen formation. The lessened ability of the reduced ferredoxin to proceed along its usual path then results in more NADH being available in the cell and since this must be recycled, it is consumed by reducing the acetyl-CoA by the only means available, since it can not reduce iacetyl-CoA directly due to a lack of aldehyde-alcohol dehydrogenase (e.g.adhE). The means by which it can get rid of the NADH and recycle this cofactor then is through greater conversion of the acetyl-CoA to acetoacetylCoA, betahydroxylbutyrylCoA, crotonylCoA and butyrylCoA. This route is already operating to a good extent in the cell since almost equivalent molar amounts of acetate and butyrate are being formed during the normal acid phase culture and this proportion of acids gives a hydrogen to carbon dioxide ratio of approximately 1.36 in the pre TNT culture (as measured by Sund and in previous literature). This pathway for acetyl-CoA allows consumption of 2NADH per molecule of butyrate formed and relieves the cofactor imbalance between ferredoxin and NADH since NADH is the reductant form used in the reduction reactions of this pathway. This also points to the relative ability of TNT to both be reduced by hydrogenase and the role it and/or a subsequent reduction product of TNT might have on inhibiting normal hydrogenase function with reduced ferredoxin. In this way the addition of TNT effectively inhibits the hydrogenase while it acts as a competing substrate and leads to built-up and redistribution of the substrate for the hydrogenase (i.e. reduced ferredoxin). A further analysis of the metabolism was also conducted. The detailed redox calculations analyzed the redistribution of reductant from production of hydrogen to more production of butyrate during the time when TNT was present and being reduced. The level of TNT present (400 μM) would not consume much of the reductant available directly in this experiment, and the amount of reductant directly consumed by the TNT would be expected to be 1 mM or less based on the early reduction steps of TNT under these conditions as analyzed previously (Ahmad and Hughes 2000; Khan et al. 1997; Padda et al. 2000). Calculations of the observed reductant consumed in the formation of acids either before or during TNT transformation and the resulting reducing equivalents available for formation of hydrogen give values of 1.30 for the hydrogen/CO2 ratio before TNT addition and 1.05-1.12 during TNT transformation. These values are very close to the values measured experimentally, before TNT addition of 1.36 and during TNT transformation of 1.18. If the data are presented in a form where the fraction of total reductant formed from glucose metabolism, i.e. reduced ferredoxin and NADH, that goes to either formation of hydrogen or butyrate is calculated, we can analyze the proportion of reductant that is consumed in butyrate formation in the culture during the one hour period before TNT addition and it is 35% of the total reductant. If the same calculation is done on the culture during the one hour period of TNT transformation the proportion of total reductant that goes into formation of butyrate is determined to be 44-47%, a significant shift of metabolism.

## 2.4 Redox mediators and iron can affect TNT degradation rate

Redox mediators are the compounds that speed up the reaction rate by shuttling electrons between electron donors and receptors. It was known that redox mediators could transfer electrons among soil microorganisms and can improve the degradation of aromatic compounds degradation and/or influence the cell physiology and metabolite pattern. Riboflavin is a typical redox mediator that is made by clostridia under certain culture conditions and has been used widely in various redox studies. However, no effect on the TNT degradation rate was observed when 100 μM riboflavin was added in the C. acetobutylicum culture assay system. Using a previously constructed riboflavin overexpression strain didn't improve the TNT degradation rate over that of wild type.

Methyl viologen is another well known redox mediator. However, the TNT degradation rate was reduced greatly when 1 mM methyl viologen was added in the assay system. TNT seems to be a facile electron acceptor and may not benefit from the presence of another transfer species which are effective on other reductive biodegradation associated processes.

Besides its rapid degradation of TNT, C. acetobutylicum was found to be able to decolorize azo dyes such as methyl red. When 200 μM methyl red (final concentration) was added into actively growing C. acetobutylicum cultures, it was discolored immediately. It was confirmed that the decolorization was due to cell metabolism since the cell free culture filtrate doesn't decolorize dye. These results indicate that C. acetobutylicum could be potentially used for more environmental bioremediation process. Examination of the metabolic products when dyes were added indicated that dyes such as viologens lead to higher butanol levels but did not seem to change the butyrate to acetate ratio at least in the experiments we have analyzed so far, in literature studies methyl viologen altered the ratio in chemstat cultures of cells poised to enter the solvent pahse. This interpretation fits with the finding of Watrous (2003) and shown again here in that hydA antisense lowered the expression and activity of hydrogenase and TNT reduction activity, while increasing the amount of hydrogenase through ovrexpression did not dramatically affect the rate of TNT transformation in cells since the limitation on reducing capacity seems to be the availability of reduced ferredoxin to transfer reducing equivalents to hydrogenase or TNT. This interpretation also fits with the lower activity of the cells later in the culture when there is more diversion of reduced ferredoxin to NADH production by the increased level of the ferredoxin-NADH reductase which lowers the amount of reduced ferredoxin available for interaction with hdrogenase. The results of previous studies on the ferredoxins of C. acetobutylicum and the level of the NADH-ferredoxin reductase and the ferredoxin-NAD reductase in cells grown under different conditions are consistent with this explanation.

A review of appropriate literature supports this proposed mode of action. The addition of the redox mediator AQDS has been

shown to lead to increased reduction of azo-dyes (e.g. reactive red 2) by anaerobic sludge and this increased reduction has been observed upon addition of riboflavin and other mediators. Neutral red served as a mediator in Clostridium acetobutylicum that coupled electron flow to produce more butanol. It was proposed methyl viologen carried e between pyr/Fd oxidoreductase and Fd-NAD oxidoreductase and the effects of dyes and iron limitation on metabolites produced by C. acetobutylicum was studied by Soucaille's group.
In our studies an number of redox dyes that may be present in soils and thus affect TNT degradation by clostridium were examined. These are listed below.

| Redox dye | Redox potential | Effect on butyrate levels |
| --- | --- | --- |
| Methylene blue | +0.011 | no |
| phenazine-1-carboxylic acid | -0.116 | More butyrate |
| TNT | -0.253 | More butyrate |
| Neutral red | -0.325 | no |
| Methyl red | -0.395 | no |
| Methyl viologen | -0.430 | No toward butanol in later cultures |
| Reference compounds | | |
| NADH | -0.320 | |
| ferredoxin | -0.390-0.410 | |
| Acetoacetyl-CoA | -0.240 | |
| Crotonyl-CoA | -0.010 | |
| Formate/CO2 | ~ -0.3 at pH 5-7 | |

These findings of the effect of competition of other factors and intracellular pathways on TNT reduction coupled with the observation of the effect of other electron carriers on the rate of TNT reduction pave the way for more detailed investigation of the effects these parameters play in defining the rate of TNT degradation in the natural environment. This is particularly interesting in light of the analysis of natural mobile soluble electron carriers in natural soil ecosystems where molecules such as quinones, and phenazines are made and released by a variety of soil organisms. Thus to define the "real world" effectiveness of various microbial communities to degrade TNT not only do we need to consider the major groups of direct degraders such as clostridia and their substrates (sugars, and polymeric substrates such as cellulose) but also consider the community population which may include those organisms which form molecules that can act as e-carrier competitors to the reaction.
Our studies with formate and clostridium showed that formate is a potent inhibitor of C. acetobutylicum. We also cloned a previously used soluble formate dehydrogenase in E. coli and then placed this gene on a clostridial expression vector and introduced it into C. acetobutylicum. We anticipated, that in the presence of formate, this type of engineered clostridial cell could provide additional reductant to the cell as it did with E.coli, and thus lead to more availability of reductant for reducing of TNT. However, this was not observed. The actions of formate and FDH in clostridium are unclear.

2.5 Analysis and interpretation of microarray data from clostridial-TNT experiments

Further microarray experiments were carried out in collaboration with Dr. Christopher Sund, at the Army Research Laboratory. TNT was added in strain 824, 824(buk), 824(pta), mutant B and M5 cultures when the culture OD600 reached 0.3. Samples were taken at the time before adding TNT, 10 minutes after TNT, 30 minutes after TNT and one hour after TNT addition. Samples of mRNA were prepared and processed for microarray analysis. The samples were quick frozen and sent for processing and analysis by Dr. Sund. Based on the data received so far we only observed a few genes were up-regulated for several fold. The responding genes were most pronounced at 10 minutes after TNT addition. These included some reductases (hydroxylamineamine reductases CAC2750, CAC3428; ferredoxin CAC2449 and ferredoxin-like protein CAC0885; 2-oxoacid:Fd oxidoreductase CAC2459; nitroreductase CAC0850; glutaredoxin CAC2777. There were also a number of redox related proteins affected and up regulated thioredoxin and thioredoxin reductase CAC3083 & CAC3082; ruberythrin CAC3597 & CAC3598; stress associated proteins cyclopropane fatty acid synthase that is induced when cells enter stationary phase and are not making hydrogen, heavy metal proteins CAC3554 & CAC3655;It is noteworthy to mention most upregulated genes were found in 10 minutes after TNT addition samples rather than in the one hour samples. The riboflavin pathway genes (CAC0590, CAC0591) were found to be up-regulated. There are some uncharacterized genes were found to be upregulated, such as putative transcriptional regulators CAC3656, CAC0876, CAC2236, CAC2242. We are studying the pattern further to see if a series of stress responsive genes were also affected.

2.6 Reduction of TNT by other clostridial strains

C. acetobutylicum has been studied as an example of clostridial species. However, there is a large group of clostridia abundant in nature, such as C. tetani, C. pasteurianum, C. kluyveri, C. aminobutyricum, C. puniceum, C. cellulolyticum, C. butyricum, C. phytofermentans, and C. cellulovorans. Many of them are capable of rapid utilization of biomass existing in natural sites that are contaminated with nitroaromatics. To further explore the action of these naturally abundant clostridia in TNT degradation and compare genomic features and differences with our previously studied C. acetobutylicum will provide clues for better understanding the potential and variability in clostridial degradation of TNT in different environments containing biomass from different sources.

We have analyzed a few of these clostridial species and one of the most interesting is Clostridium phytofermentans, that is used to degrade cellulose and produce the fuel, ethanol. This strain was grown on cellulobiose and the reaction of TNT was studied. It was found that TNT was reduced readily, however the maximal rate was not as fast as with C. acetobutylicum or related strains growing on glucose. It was especially interesting since the strain normally prodces ethanol as the reduced product and thus might be expected to act in TNT reduction as if it were in the solvent phase, a phase where C. acetobutylicum exhibits low TNT reducing activity. This finding broadens the group of anaerobes that can play a role in the degradation of TNT in natural sites that have cellulosic substrates available and this would be favorable since these materials are low cost and often abundant in sites.

### 2.7 Efforts at reconstitution of the clostridial system in E. coli for further study

We have obtained and prepared strains of E. coli that should be useful in analyzing the TNT transformation process in more detail. These components are: a pyruvate-ferredoxin oxidoreductase from Desulfovirbrio africanus and various ferredoxin constructs (Pieulle et al J. Bact 179, 5684-5692, 1997; Agapakis et al J. Biol Engineering 4, 3, 2010), a Clostridium acetobutylicum hydrogenase and hydrogenase maturing protein expression construct (King et al J. Bact 188, 2163-2172, 2006; Akhtar & Jones Anal Biochem 373, 170-172, 2008). These coupled with our existing E. coli mutant strain assortment, can allow further details of the redox reactions involved in TNT transformation to be manipulated and examined.

### 2.8 Study of phenazine as an analog of TNT to show the generality of the discovery of TNT effects on metabolic pattern and possible complications of soil electron mediators on TNT degradation.

We observed that phenazine carboxylate was a reasonable derivative to test in terms of solubility and the formation of this compound in nature by soil microbes. Therefore it was of interest to test as in soils where this compound or similar compounds exist they could significantly affect the ability of clostridia to degrade the TNT. This inhibition which would prolong or eliminate the ability of normal anaerobic remediation of soils contaminated with TNT would be an important factor to analyze in future assessment of the potential for soil communities to degrade TNT. The examined the concentration of phenazine carboxylate required to show a similar metabolite change in clostridial cultures as was found in the TNT cultures (section 2.3). Two test strains of clostridium were investigated. These were the wild type Clostridium acetobutylicum ATCC824 and a mutant M% which does not form solvents and as such is more like the acid forming clostridia like C. butyricum. In both cases the butyrate to aectate ratio was increased at rather low concentrations of the phenazine compound indicating that the electron interactions of the compound were able to increase the proportion of available redox going to NADH, the reduced cofactor required to form the longer chain organic acid, butyrate. This removal of some reducing power from the ferredoxin to NADH then removes the ability of the reduced ferredoxin to interact with hydrogenase where, it seems, most of the TNT transformation happens. A preliminary experiment with some redox compounds and TNT indicated a lower extent of TNT transformation in the presence of the mediators.

### 2.9 Summary of implications of the work.

These findings are summarized briefly:

1. TNT transformation can account for a high proportion of cell redox metabolism
2. Many clostridial species including cellulolytic can degrade TNT quite well
3. TNT transformation alters the metabolic product profile in a novel fashion, toward longer chain acids
4. We tested and found certain other redox active dyes (some naturally occurring in soil environments) also generate this change in acid profile of anaerobic fermenters

Implications for TNT remediation by soil communities

1. The presence of iron chelators and redox active molecules in soil can greatly affect the TNT transformation rate even if degrading organisms and substrates are present
2. The measurement of these compounds and the organisms that produce them (in addition to the levels of clostridia and substrate) is likely to provide useful information for estimating potential TNT degradation rates in situ.

Implications for science and biochemistry in a broader context

1. The presence of various redox active substances can perturb the metabolic pattern of microbes by affecting cofactor balance
2. The modulation of the relative flow between the redox cofactors; the NADH and ferredoxin carriers, can affect metabolism in previously unforeseen and dramatic ways
3. In biotech this could be important in forming longer chain length molecules desired as biofuels
4. In health, butyrate is a key molecule for proper development of the intestinal epithelium and the gut immune system, as well as having wider positive effects in the human body: thus ingestion or production of such redox active molecules could have significant health effects by disturbing or improving the pattern of short chain fatty acids in the gut.

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## Technology Transfer

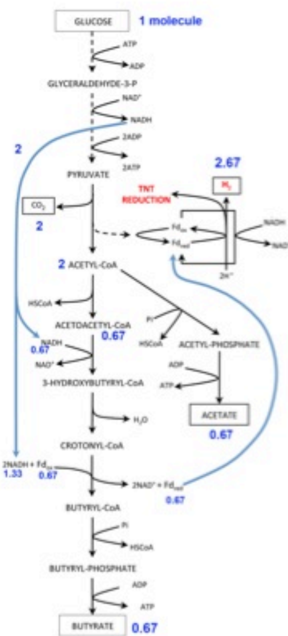
Attachment to Final Report

The diagram below shows the flow of electrons (as redox equivalents) during the growth of *Clostridium acetobutylicum* in normal acidic phase culture and when TNT is added to the culture. The amount of ferredoxin in the reduced form going to hydrogen is lowered in the TNT treated culture, and while some of the redox goes directly to TNT transformation to form the degradation products, this does not account for the change of redox going to hydrogen. The less amount of reduced ferredoxin in the case of the TNT treated culture requires that the reduced ferredoxin goes into other reactions in the cell. In this culture state, that of acid production and not alcohol, has the possibility to use the redox in the formation of longer chain length acids such as butyrate where the reduced ferredoxin can be used with NADH to form the longer chain through the electron transfer factor proteins and the bifrucating enzyme. This avenue is not available is acetate is formed since it does not require any redox in formation from acetyl-CoA. The quantitative flux pattern was determined from measurements of the organic acids formed, the glucose utilized and the hydrogen formed during the normal culture and the TNT treated culture and reflects all the information in a global picture and is consistent with the normal patterns of clostridial fermentations in the early acid forming stage.

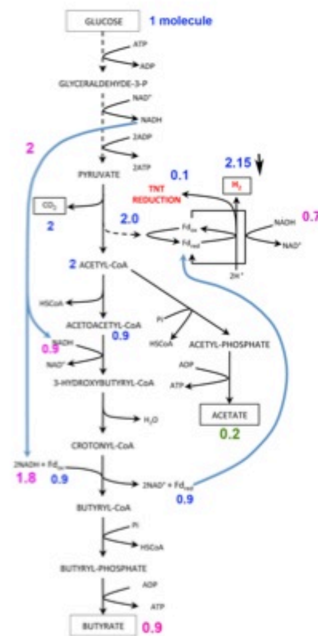
Figure 1.Diagram comparing the redox flux in *Clostridium acetobutylicum* cultures with and without TNT addition.

Increased butyrate & cofactor imbalance when $H_2$ formation inhibited

**Normal acid phase pattern**




**Pattern with TNT**



In the below figure, the effect of phenazine carboxylate on metabolite pattern of Clostridium acetobutylicum is shown. Further studies with early cultures of the solvent forming strain and a non-solvent forming mutant were conducted and the similar change in the butyrate to acetate ratio was found. We are planning a subsequent publication of this finding.

Figure 2. The effect of phenazine, a compound with a similar rredox potential to TNT, on the metabolic pattern of *Clostridium acetobutylicum*.

**Phenazine carboxylic acid gives a high butyrate to acetate ratio in CGM and buffered CGM**