

## Reductive Dehalogenation of Trichloroacetic Acid by *Trichlorobacter thiogenes* gen. nov., sp. nov.

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**A bacterium able to grow via reductive dechlorination of trichloroacetate was isolated from anaerobic soil enrichments. The isolate, designated strain K1, is a member of the  $\delta$  proteobacteria and is related to other known sulfur and ferric iron reducers. In anaerobic mineral media supplemented with acetate and trichloroacetate, its doubling time was 6 h. Alternative electron donor and acceptors were acetoin and sulfur or fumarate, respectively. Trichloroacetate dehalogenation activity was constitutively present, and the dechlorination product was dichloroacetate and chloride. Trichloroacetate conversion seemed to be coupled to a novel sulfur-sulfide redox cycle, which shuttled electrons from acetate oxidation to trichloroacetate reduction. In view of its unique physiological characteristics, the name *Trichlorobacter thiogenes* is suggested for strain K1.**

Trichloroacetic acid (TCA) is an industrial chemical that is found in nature mainly through its use as a herbicide against perennial weeds. Decomposition of TCA in soil seems to be slow (9) and is mainly microbially mediated. However, little is known about the microorganisms and processes involved. In the experiments of Hirsch and Alexander (7) a *Pseudomonas* isolate, actively degrading 2,2-dichloropropionic acid, also showed activity on TCA. Jensen (8) and Lode (10) reported on *Pseudomonas*, *Arthrobacter*, and *Agrobacterium* species and on an *Arthrobacter* strain, respectively, that were all able to split off chlorine from TCA in a dissimilatory process. In a recent study by Yu and Welander (19), the first aerobic bacterium able to grow on TCA as the sole carbon and energy source was characterized. The isolate belonged to the  $\gamma$  subgroup of proteobacteria and could not grow on mono- and dichloroacetic acid (MCA and DCA). Although aerobic TCA decomposition has been recognized for a few decades, its anaerobic degradation has not been demonstrated. We now report on the isolation of a bacterium capable of anaerobic growth via reductive dehalogenation of TCA, and we propose that a sulfur-sulfide redox cycle is involved in this process.

### MATERIALS AND METHODS

**Isolation and maintenance of strain K1.** Anaerobic medium was prepared under oxygen-free  $N_2$ - $CO_2$  (80:20) as described by Cole et al. (3) and consisted of a basal salts medium amended with vitamins and a  $Na_2SeO_3$ - $Na_2WO_4$  solution (3). As a reductant, 1 mM  $Na_2S$  was added to the medium after it had been boiled and cooled to room temperature. The pH was adjusted to 7.2 by varying the flow of  $CO_2$ . Except for acetate, which was added before boiling the medium, electron donors, electron acceptors, and vitamins were added from sterile anoxic stock solutions after autoclaving.

TCA-degrading organisms were enriched from four subsoil samples from each of two sites. Site K, located along Mill Creek in Muskegon County, Mich., was formerly a Koch Industries chemical processing plant and is a Michigan Superfund site. Several chlorinated chemicals leached through soils of this site. Site M, located east of Grant in Newago County, Mich., is a muck soil site that was drained in the 1930s and is used for vegetable production. Chlorinated pesticides have been used on this soil. A composited soil sample from each site was mixed in a 1:10 ratio with anaerobic medium amended with 1 mM TCA and 2.5 mM acetate. The 100-ml enrichment cultures were incubated in 160-ml serum bottles

at 25°C and regularly monitored for TCA concentrations by high-pressure liquid chromatography (HPLC). When TCA had disappeared from the culture supernatant, 1 mM TCA was added. After several TCA additions, 10% and then 1% transfers to fresh enrichment medium were made, totalling 11 serial transfers over a 7-month period. Finally, these cultures were diluted 1:10 to extinction to obtain pure cultures.

For maintenance, the isolates were kept on anaerobic medium amended with 2.5 mM of acetate and 1 mM TCA. After the conversion of four to five TCA additions, 1% inocula were transferred to fresh medium and again incubated at 25°C. Likewise, 1% inocula were used in the experiments described below.

**Identification of strain K1.** Strain K1 was selected for further study. To confirm its purity, 1% inocula were transferred to fresh anaerobic medium with different electron donor-electron acceptor combinations that supported growth of K1. These were acetate-sulfur, acetate-TCA, or acetate-fumarate (see Results). After growth at 25°C, cells were harvested, washed twice with 1 M NaCl, and resuspended in 50  $\mu$ l of MilliQ water. Aliquots were then subjected to BOX-PCR according to the method of Rademaker et al. (15), and the genomic fingerprints were compared.

For identification purposes, the 16S rRNA sequence of strain K1 was determined essentially as described by Cole et al. (3). The following aligned rRNA sequences were obtained from the Ribosomal Database Project (RDP) (13) release 7.1 (GenBank accession numbers are in parentheses): *Desulfuromusa succinoxidans* (X79415), *D. kysingii* (X79414), and *D. bakii* (X79412); *Malonomonas rubra* (Y17712); *Pelobacter acidigallici* (X77216); *Desulfuromonas thiophila* (Y11560) and *D. acetoxidans* (M26634); *P. carbinolicus* (X79413); *P. acetylennicus* (X70955); *Desulfuromonas palmitatis* (U28172); *Geobacter sulfurreducens* (U13928), *G. "hydrogenophilus"* (U28173), and *G. metallireducens* (L07834); *Pelobacter propionicus* (X70954); *G. "chappelleii"* (U41561); *Desulfuromonas* sp. (M80618); and *E. coli* (J01695). The sequence of strain K1 was added to this alignment with the Sequence Aligner tool offered at the RDP web site. Unambiguously aligned regions (1430 positions) were marked using the program GDE (17). These positions were used to create an unrooted maximum-likelihood phylogenetic tree (5) with the program fastDNAm1 (14). Phylogenetic-tree analysis was repeated on 100 bootstrap samples (6). Numbers at internal nodes indicate the number of times out of the 100 bootstrap samples that the cluster defined by the node was monophyletic. Organism names and type status are from RDP release 7.1.

**Substrate use by strain K1.** To evaluate the electron donor and electron acceptor specificity of strain K1, duplicate cultures were grown in 160-ml serum bottles containing 100 ml of anaerobic medium. All cultures were inoculated with a 1% transfer from a liquid culture grown on acetate-TCA. Uninoculated controls and references without electron donors or acceptors were included, and incubations were performed quiescently at 25°C. Different substrates were tested for their potential to support 1 mM TCA dechlorination by strain K1. The following compounds were selected: acetoin (10 mM), glycerol (10 mM), ethylene glycol (10 mM), ethanol (10 mM), butyrate (1 mM), propionate (1 mM), acetate (1 and 2.5 mM), formate (5 mM), lactate (10 mM), thiosulfate (5 mM), ferric citrate (5 mM), and  $H_2$  (10% in the headspace). At regular time intervals, samples were taken and centrifuged, and the supernatant was monitored for TCA disappearance by HPLC analysis.

Alternative electron acceptors were tested using 1 mM acetate as the electron donor: trichloroethene (18  $\mu$ mol/160-ml vial), trichloroethane (18  $\mu$ mol/160-ml vial), TCA (1 to 10 mM), DCA (1 mM), MCA (1 mM), trifluoroacetate (1 mM), fumarate (20 mM), pyruvate (10 mM), and elemental sulfur.

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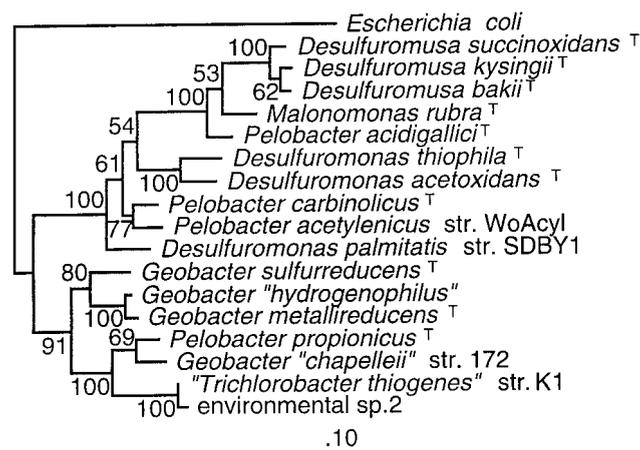


FIG. 1. Phylogenetic tree based on the 16S rRNA sequences of strain K1 (*T. thiogenes*) and representative bacteria.

**TCA dechlorination by strain K1.** To determine whether the enzymes necessary for TCA dechlorination are constitutively expressed or induced by TCA, an induction experiment was performed. Cultures were grown in parallel on acetate-sulfur and on acetate-TCA-sulfur media. After conversion of TCA to DCA in the latter culture, both sets were fed 0.5 or 1 mM TCA. They were sampled over several hours, and the culture supernatants were monitored for TCA dechlorination.

For resting cell experiments, cells grown in acetate-TCA amended anaerobic medium were collected on 0.22- $\mu$ m (pore-size) filters and resuspended in buffer, acetate-containing medium, or filtered supernatant. After the addition of 1 mM TCA, the TCA dechlorination activity was monitored in comparison to respective controls without cells.

To study TCA dechlorination in detail, a modified chloride-free anaerobic medium was prepared which had the same composition as the basal salts medium except that the chloride salts were replaced with the corresponding sulfate salts. In addition, the reducing agents were 0.2 mM concentrations of both L-cysteine and Na<sub>2</sub>S<sub>9</sub>H<sub>2</sub>O instead of 1 mM Na<sub>2</sub>S<sub>9</sub>H<sub>2</sub>O alone, since high sulfide concentrations interfere with chloride determinations (4). After the addition of a 1% inoculum to the medium, samples were taken at regular intervals to monitor the TCA, DCA, MCA, and chloride concentrations.

A second-generation stoichiometry experiment was set up to check the hypothesis of a sulfur-sulfide redox cycle being involved in TCA dechlorination. After the conversion of a first TCA pulse in inoculated acetate-TCA amended anaerobic medium, a second TCA pulse was added. From then on, samples were taken at regular intervals and analyzed for acetate, TCA, DCA, sulfur, and sulfide concentrations. Uninoculated blanks and references to which no second TCA pulse was given were provided. The experiment was performed in duplicate and repeated four times.

**Analytical methods.** The disappearance of TCA and other haloalkanoates was monitored by HPLC analysis (Hewlett-Packard HPLC 1050; Microsorb MV Amino column [25 cm]; 60:40 25 mM phosphate buffer [pH 3.8]-acetonitrile eluents; flow rate, 1 ml  $\cdot$  min<sup>-1</sup>; UV detection at 210 nm). Trichloroethane and trichloroethene were measured in headspace samples by gas chromatography (GC) (11), and nonhalogenated organic acids were measured by HPLC (12). Sample volumes for GC and HPLC analysis were 200 and 50  $\mu$ l, respectively. HPLC analyses were performed on the supernatants of centrifuged culture samples (5 min, 10,000  $\times$  g).

Chloride release was measured colorimetrically according to the method of Bergmann and Sanik (2). Sulfide was determined according to the methylene blue method (18). Quantification of sulfur in culture medium was performed as follows. A 5-ml sample was filtered over a 0.45- $\mu$ m (pore-size) nylon membrane. The filter was washed with cold water, dried, and then soaked in ethanol to redissolve the precipitate. Absorption was measured at 264 nm, and sulfur concentrations were determined using elemental sulfur dissolved in ethanol as a standard.

Specific peaks collected during HPLC runs of culture supernatant were derivatized to their propyl esters (16) and subjected to GC-mass spectrometry (MS) analyses in the electron impact, selected ion monitoring mode (SPB20 column). Selected *m/z* values were 107 and 137 for MCA and 111, 141, and 171 for DCA.

The 16S rRNA sequence has been deposited under GenBank accession no. AF223382.

## RESULTS

**Isolation and identification of TCA-degrading organisms.** As anaerobic enrichments of soil samples were repeatedly trans-

ferred to fresh medium, soil-free cultures were obtained with stable TCA-degrading properties. Serial 1:10 dilutions to extinction finally yielded pure cultures which consisted of strictly anaerobic, gram-negative short curved nonmotile rods. Although the liquid cultures never appeared turbid, cells were present in high densities. Tenfold dilutions from a 100-ml culture into balchtubes containing 9 ml of acetate-TCA amended medium still showed TCA dechlorination at dilutions up to 10<sup>-8</sup>. Vitamins were not required for growth on acetate-TCA in the basal salts medium, and no growth was ever detected on complex media, on solid media, and in aerobic conditions.

Two isolates, strains K1 and M1, were obtained from the two different sites. They had similar morphology and acetate-TCA growth characteristics, but different rep-PCR patterns. One isolate, K1, was selected for further study. It appeared pure by microscopic observation. In addition, cells grown on different electron donor-electron acceptor combinations yielded identical BOX-PCR patterns, which is strong evidence that K1 is a pure culture. Phylogenetic analysis of strain K1's 16S rRNA sequence shows that it is a member of the  $\delta$  proteobacteria and that it falls in a cluster of mixed taxonomic affiliation (Fig. 1). Interestingly, it appears to be most closely related (99% sequence identity) to a clone presumably reflecting an unisolated organism from a sulfate-reducing bioreactor (1). Among isolated bacteria, its closest relatives are *G. "chapelleii"* and *P. propionicus* (94% sequence identity to each).

**Substrate use by strain K1.** Strain K1 was isolated on 2.5 mM acetate-1 mM TCA amended anaerobic medium. As alternative electron donors, substrates were selected which supported the growth of related *Pelobacter* species. In the experimental conditions used, only acetate and acetoin amendment led to TCA disappearance, and TCA dechlorination always stopped at the level of DCA. As for alternative electron acceptors, none of the halogenated compounds other than TCA was transformed by strain K1 in the presence of acetate as an electron donor. However, acetate consumption was observed in the presence of sulfur or fumarate as the electron acceptor. In the latter case, succinate was detected as the product of fumarate reduction.

As can be seen from Fig. 2, strain K1 typically converted TCA into DCA with the concomitant consumption of acetate. For 2.5 mM acetate in basal salts medium, 10 additions of 1 mM TCA were dehalogenated at maximum, and at that point, acetate was exhausted from the medium. When lower acetate concentrations were provided, 1 mM TCA was completely converted to DCA only at acetate levels exceeding 0.25

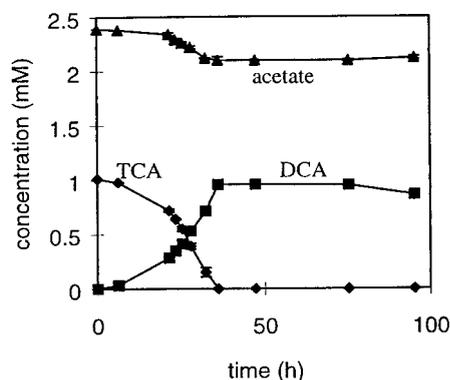


FIG. 2. Transformations during growth of strain K1 in basal salts medium with 2.5 mM acetate and 1 mM TCA. Symbols: ▲, acetate; ◆, TCA; ■, DCA.

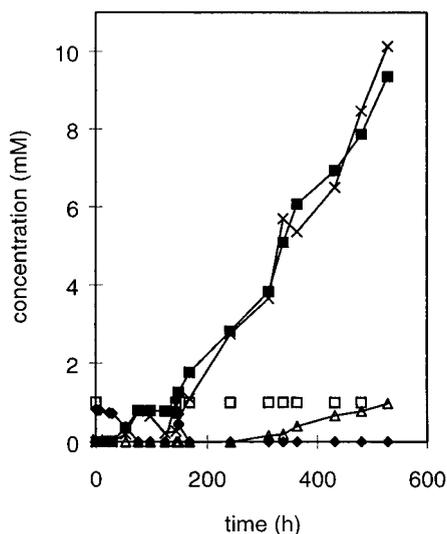


FIG. 3. Dechlorination of subsequent 1 mM TCA pulses by strain K1 in a chloride-free medium. Symbols: □, TCA additions; ◆, TCA; ■, DCA; △, MCA; ×, chloride.

mM. At 0.1 mM acetate, dechlorination stopped but resumed when additional acetate was provided.

To confirm the formation of DCA as an endproduct of TCA dechlorination, strain K1 was inoculated in chloride-free medium amended with 2.5 mM of acetate and 1 mM TCA. Figure 3 illustrates that the production of DCA and chloride was similar and that, on a molar basis, their concentration increase was equal to TCA disappearance. In four independent trials, DCA and chloride were produced in equimolar amounts. HPLC analysis also indicated the accumulation of MCA in one of the replicates starting at the fourth TCA addition. The suspected DCA and MCA peaks were collected, derivatized to propyl esters, and subjected to GC-MS analysis in selected ion monitoring mode. The occurrence of peaks at  $m/z$  values specific for DCA and MCA unambiguously proved the presence of both dehalogenation products in the collected fractions.

**TCA dechlorination by strain K1.** From resting cell assays, it was clear that filtered supernatant of spent medium did not show any TCA dechlorination activity. Reduction of TCA required cells and an electron donor, in casu acetate. However, TCA dechlorination was faster when cells were resuspended in supernatant than when resuspended in fresh medium. In the cell-free controls, no TCA disappearance was observed.

The generation time of strain K1 on acetate-TCA was determined from direct cell counts using acridine orange and was estimated to be between 5 and 6 h. TCA concentrations of up to 10 mM were still dehalogenated. Induction experiments showed that TCA dechlorination did not proceed at a faster rate in cultures which had previously been exposed to TCA.

When a new TCA pulse was added to an acetate-TCA-grown culture immediately after the complete conversion of the previous pulse, the transient formation of a precipitate was visually observed in the medium. Microscopic investigation indicated the appearance of refractile spheres, sometimes associated with the cells shortly after TCA was added (Fig. 4). Since the refractile spheres were reminiscent of elemental sulfur, perhaps formed by oxidation of the sulfide reducing agent, a second-generation stoichiometry experiment was set up to monitor both sulfur and sulfide concentrations after respiking TCA. TCA conversion to DCA was accompanied by acetate consumption and by the temporary oxidation of sulfide to sul-

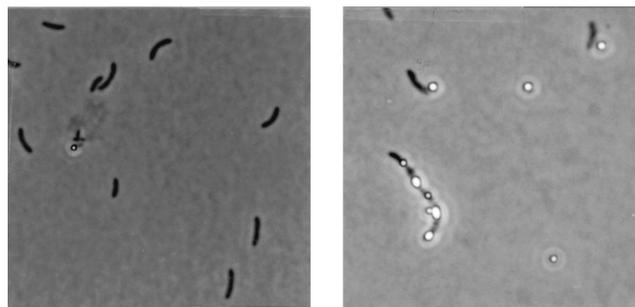


FIG. 4. Microscopic image of strain K1 grown in basal salts medium with acetate and TCA after conversion of the first TCA pulse (left) and 1 h after the addition of a second TCA pulse (right). The refractile spheres are sulfur granules.

fur (Fig. 5). When TCA concentrations were nearly exhausted, the original sulfide concentrations were reattained. Similar observations were made in four independent incubations. The fact that the sulfur stoichiometry was not complete was probably due to the incomplete retention of sulfur on the filtration membrane, the unaccounted presence of intracellular sulfur, or on the formation of polysulfides in the medium.

## DISCUSSION

The ability of TCA to support the growth of microorganisms had been questioned until Yu and Welander (19) demon-

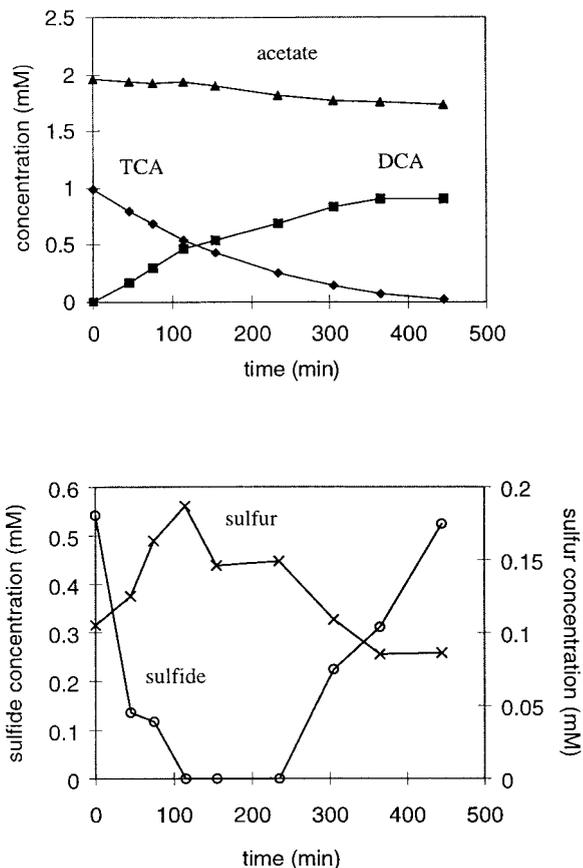


FIG. 5. Concentration profiles after the addition of a second 1 mM TCA pulse to basal salts medium amended with acetate. Sulfide was the reducing agent in the medium.

strated the existence of aerobic bacteria capable of using TCA as the sole source of carbon and energy. In contrast, TCA degradation in anaerobic conditions has not yet been described. Only one report was found which mentions TCA disappearance in a reactor with an immobilized methanogenic mixed culture, but the authors ascribe TCA transformation to a combination of chemical and biological reactions (4). The isolation of a bacterium able to grow via reductive TCA dechlorination clearly establishes the existence of an anaerobic TCA removal process. Anaerobic soil enrichments on acetate-TCA yielded two similar isolates, of which strain K1 was further characterized.

The new isolate appears to gain energy from the dechlorination reaction. Numerous 1% serial transfers into anaerobic medium amended with acetate and TCA consistently led to TCA disappearance and DCA production, along with an increase in cell number to densities of at least  $10^8 \cdot \text{ml}^{-1}$ . By varying the molar ratio of acetate to TCA in the medium, the stoichiometry of TCA was indicated to be as follows:  $\text{CH}_3\text{COOH} + 4 \text{CCl}_3\text{COOH} + 2 \text{H}_2\text{O} = 2 \text{CO}_2 + 4 \text{HCl} + 4 \text{CHCl}_2\text{COOH}$ . The growth of strain K1 in chloride-free medium and the measurement of chloride release confirmed the proposed stoichiometry (Fig. 3). Why chloride concentrations decreased after conversion of the first TCA pulse, is unclear, but is probably attributable to experimental error. In any case, four independent experiments demonstrated the presence of equimolar amounts of DCA and chloride, which strongly suggests that TCA dechlorination stops at the level of DCA. In one instance only, a gradual MCA accumulation was evidenced by HPLC and GC-MS analysis. Although MCA production could never be reproduced, strain K1 may have the capability to dechlorinate TCA to MCA under certain conditions. Further research will be necessary to elucidate which factors are responsible for a limited dehalogenation and/or which factors can trigger a twofold dechlorination. In further support of this suggestion, we observed that in some soil microcosms TCA was converted to DCA when acetate was provided as an electron donor, while TCA was completely dehalogenated in the presence of other electron donors. This was, however, not the case with strain K1. Whether TCA dechlorination proceeds to DCA or MCA does not alter the fact that strain K1 most probably gains energy by using TCA as a respiratory electron acceptor.

In addition to the fact that it is an unusual and constitutive reaction, TCA dehalogenation also seems to have high substrate specificity. Indeed, none of the alternative halogenated compounds tested was transformed by strain K1. Only elemental sulfur or fumarate could serve as electron acceptor for acetate oxidation. The results for ferric iron reduction were not consistent. Therefore, it appears that strain K1 has an extremely narrow electron donor and acceptor specificity. Its ability to use sulfur as an electron acceptor is not surprising in view of its phylogeny. Based on its 16S rRNA sequence, it is placed among a group of microorganisms in the  $\delta$  subdivision of the proteobacteria, which are known ferric iron or elemental sulfur reducers.

In strain K1, sulfur reduction seems to be connected to TCA dechlorination in a novel redox cycle. In all experiments, respiking of TCA to acetate-TCA-grown cultures induced the transient formation of a precipitate in the medium. This phenomenon apparently depended on the physiological state of the microorganisms and on the medium composition. No precipitation was seen when the time lag between TCA depletion and readdition was too long. Neither did the medium turn turbid upon a second TCA addition, when the reductant sulfide was replaced by L-cysteine. Stoichiometry experiments confirmed that TCA respiking led to the temporary oxidation

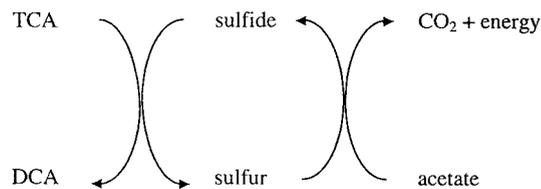


FIG. 6. Proposed sulfur-sulfide redox cycle as electron transmitter between acetate and TCA.

of sulfide to sulfur followed by its reduction to sulfide at the time TCAA was nearly exhausted (Fig. 5).

To explain these experimental observations, we hypothesize that a sulfur-sulfide redox cycle is involved in TCA conversion. As visualized in Fig. 6, electrons are transferred in a first step from acetate to sulfur, yielding sulfide, CO<sub>2</sub>, and energy. The sulfide-reducing equivalents are then used in TCA dehalogenation to DCA, thereby closing the sulfur-sulfide cycle. The observed temporary appearance of sulfur in the medium implies that the TCA-sulfide reaction is faster than the sulfur-acetate reaction. The following observations are all in accordance with the proposed mechanism. Strain K1 is a sulfur reducer and oxidizes acetate in an acetate-sulfur medium, either in the absence or presence of sulfide. When incubated with acetate and sulfide, no sulfur production or acetate consumption takes place, but when strain K1 was incubated with sulfide and TCA, partial TCAA dechlorination was noticed. Although it is not yet known how electrons are being transferred from sulfide to TCA, evidence suggests that a cell-associated catalyst and a reductant (acetate) are required. Resting-cell experiments clearly showed that the process of TCA dechlorination required actively metabolizing cells, since cells in the buffer did not mediate the reaction. The fact that the dechlorination rate was higher when cells were returned to spent medium than when resuspended in fresh medium may implicate that a secreted electron shuttle is also involved.

Other evidence suggests that the sulfur-sulfide cycle is not essential nor exclusive for TCA dechlorination. First, the sulfur-sulfide redox cycle was also found to mediate electron transfer from acetate to fumarate, since respiking of fumarate also led to sulfur precipitation. Second, parallel incubations of strain K1 on acetate-TCA in a medium with either sulfide or cysteine as a reductant, showed a similar TCA dechlorination behavior. Up to nine TCA additions were dehalogenated at the same rate, but transient turbidity due to sulfur was evident only in the sulfide-amended medium. Subculturing in cysteine-reduced medium did not affect dechlorinating activity, and in the absence of acetate no TCA conversion took place. Hence, cysteine probably had a similar electron shuttling function as proposed for sulfide. In spite of the fact that the sulfur-sulfide cycle is not essential for TCA dechlorination, it remains to be investigated how widespread this type of redox cycle is, particularly among sulfur-reducing bacteria, since they may present an as-yet-unknown potential for dechlorination activity in nature.

In summary, we have isolated a novel bacterium growing via reductive dehalogenation of TCA and also capable of sulfur reduction. Its most interesting feature is the involvement of a sulfur-sulfide redox cycle in TCA dehalogenation. The difference in 16S rRNA sequence from the closest strains in the phylogenetic tree (6%) and its unique physiological characteristics demonstrate that strain K1 represents a new genus. The name *Trichlorobacter thiogenes* is proposed, which emphasizes its capability to reductively dechlorinate TCA and its sulfur cycling activity.

**Description of *Trichlorobacter* gen. nov.** *Trichlorobacter* (Tri. chlor.o.bac'ter) L. num. *tria* three; Gr. n. *chloros* chlorine; M.L. masc. n. *bacter* the equivalent of Gr. neut. n. *bactrum* a rod, M. L. masc. n. *Trichlorobacter* because it is a TCA-dechlorinating rod.

**Description of *T. thiogenes* sp. nov.** *T. thiogenes* (thi.og'en.es) Gr. n. *theion* sulfur; M. L. n. *genes* production; M. L. n. *thiogenes* because it produces sulfur. *T. thiogenes* is a strictly anaerobic bacterium with rod-shaped cells. Cells are gram negative, short, curved and nonmotile. *T. thiogenes* grows by oxidizing acetate and acetoin with the concomitant reduction of TCA. Other halogenated compounds are not used as an electron acceptor. *T. thiogenes* can also conserve energy for growth by the reduction of sulfur and fumarate. It does not grow on complex and solidified media.

The type strain is *T. thiogenes* K1, which was enriched from subsoil from western Michigan with acetate as an electron donor and TCA as an electron acceptor.

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