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Plasmids in tributyltin-resistant bacteria from fresh and estuarine waters

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SUMMARY

Twenty-six tributyltin (TBT)-resistant bacterial strains isolated from sediments were examined for the presence of plasmids. Plasmids of the size reported to carry metal resistance genes were not found in 15 of the strains, indicating that resistance does not have to be plasmid-mediated. Attempts to cure plasmid-containing strains using acridine orange, ethidium bromide, novobiocin or sodium dodecylsulfate, or by growth at elevated temperature were not successful, nor were plasmids transferred from TBT-resistant strains into TBT-sensitive organisms by electroporation. In a broth mating experiment, however, plasmid pUM505, a conjugative plasmid known to encode chromium resistance in *Pseudomonas aeruginosa* PAO1, was introduced into TBT-sensitive *Beijerinckia* sp. MC-27 isolated from freshwater sediment. The TBT tolerance of the *Beijerinckia* sp. increased 100-fold, from 8.4 μM TBT in *Beijerinckia* sp. MC-27 to 840 μM TBT in *Beijerinckia* sp. MC-27 (pUM505) on solid medium. The plasmid was transferred at a frequency of approximately 6×10^{-4}. TBT-resistant transconjugants grew faster in media containing TBT and lost their enhanced TBT tolerance and the plasmid upon serial transfer in medium without TBT. Spontaneous mutants of the donor *P. aeruginosa* lost both TBT resistance and the plasmid. Therefore, TBT resistance in bacteria can be plasmid-mediated. To our knowledge, this is the first report that resistance to a tin compound can be plasmid-mediated.

INTRODUCTION

Tributyltin (TBT) is used as a stabilizer in plastics, as a wood preservative, and most commonly as the active component in antifouling paints. It has proven effective in controlling colonization of submerged surfaces by the zebra mussel, *Dreissena polymorpha* [18], and barnacles [7], but it is highly toxic to a variety of non-target organisms as well at levels as low as 1–10 parts per trillion (p.p.t.) Sn [14,21]. Molluscs are unusually sensitive to TBT because they have low activities of cytochrome *P*-450 and mixed function oxidases, leading to TBT accumulation in tissues since TBT is metabolized slowly [16]. Accumulated TBT in molluscs causes a significant increase in testosterone leading to a condition known as imposex, wherein female animals develop male sex organs and the population cannot reproduce [29]. This toxicity led to its regulation in a number of countries and in 1988 in the United States [32]. TBT may still be applied to vessels longer than 25 m, to aluminum-hulled boats and to such submerged surfaces as water intake pipes. Because of continuing use and slow degradation rates, TBT persists in both freshwater [19] and marine sediments [15,33,35].

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TBT is toxic to a variety of microorganisms [2,4,10,24 and reviewed in 9 and 11]. TBT-resistant organisms can be isolated readily [3,31,36]. Some microorganisms can participate in degradation of TBT to dibutyltin, monobutyltin, and inorganic tin [reviewed in 8]. Wuertz et al. [36] found that the 50% effective concentrations (EC_{50}s) for microbial populations were higher at a TBT-polluted freshwater site than at a site without detectable amounts of TBT, suggesting that TBT selected for a TBT-resistant population at the contaminated site.

The exact mechanism(s) of microbial resistance to TBT remain unclear [11]. Metal-tolerant bacteria exhibit multiple metal-resistance mechanisms, the most common of which is plasmid-mediated ion efflux [26]. Summers and Jacoby [30] and Belliveau et al. [3] did not find plasmids in Gram-negative bacteria resistant to inorganic tin. Among organometals only resistance to organomercurials is known to be plasmid-mediated [20]. Fukugawa and Suzuki [13] cloned a large chromosomal gene involved in TBT resistance in an *Alteromonas* sp., and they suggested that TBT resistance is encoded by several genes. They also report (this volume) bacteria tolerant to TBT and Cd, and to TBT and methyl mercury. We determined the plasmid content in environmental isolates which are resistant to TBT to investigate the potential roles of plasmids in TBT resistance, and we provide evidence that TBT resistance can be plasmid-mediated.

MATERIALS AND METHODS

Organisms and plasmids

Pseudomonas aeruginosa PAO1 harboring plasmid pUM505 [6] was obtained from the Idaho National Engineer-

ing Laboratory, Idaho Falls, ID, USA. *Escherichia coli* WM1100, a *recA* derivative of MC1061 with the genotype F-*araD*139Δ (*araleu*) 7696Δ (*lac*) ×74*galUgalKhsd*R2 (r_k-M_k+) *mcr*B1*rps*L (str^R) Δsrl*recA* (306::Tn10) was obtained from M.M. Doolittle, University of Massachusetts, Boston, MA, USA. *Escherichia coli* JM107 pBluescript II SK(+) was obtained from R.S. Hanson, Gray Freshwater Biology Institute, University of Minnesota, Navarre, MN, USA. All other organisms were isolated from estuarine or fresh waters and cultured as described by Wuertz et al. [36] and display a wide range of TBT resistance levels (Table 1). Isolate MC-27, previously identified as a *Flavobacterium* sp. [36], was later identified as a *Beijerinckia* sp. based on the appearance of colonies on N-free agar, the presence of polar bodies in cells, growth at pH 4 and other physiological and biochemical characters. Each isolate was screened for the presence of plasmid DNA by at least two and up to five different plasmid isolation methods (Table 1), all modifications of the alkaline lysis procedure. Additionally, several estuarine strains which appeared to be plasmid-free were examined for the presence of endogenous nucleases which could potentially degrade the plasmid DNA before it was detected by agarose gel electrophoresis: 2 ml of a culture of *E. coli* JM107 containing pBluescript SK(+) were added to 20-ml cultures of selected isolates (TBT-2, TBT-6, TBT-8, SHC-6, SHC-10, and SHC-11) and the mixed culture was extracted.

TABLE 1

Organisms used, their TBT resistance and plasmid content

Genus or genus and species-strain no.	EC_{50} of TBT on solid medium[a] (μM)	Plasmid screening methods employed[b]	Presence of plasmid DNA by any screening method (no. of plasmids)
Freshwater organisms			
Pseudonomas-NOWC1	6.87	CF, BD	0
Bacillus-NOWC2	19.3	CF,BD	0
unknown-NOWC3	1683	CF,BD	1
Pseudomonas-NOWC4	59.9	CF,BD	0
Alcaligenes-NOWC5	43.8	CF,BD	0
Alcaligenes-NOWCS	33.6	CF,BD	0
Flavobacterium-OWC7	6.3	CF,BD	2
Caulobacter-OWC8	1.4	CF,BD	0
Pseudomonas-BP1	850	CF,BD	0
Pseudomonas-BP2A	23.0	CF,BD	5
Pseudomonas-BP2B	0.8	CF,BD	1
Klebsiella-BP3	8.4	CF,BD	0
Pseudomonas-BP4	8.4	CF,BD	4
Alcaligenes-BP5	8.4	CF,BD	?
Alcaligenes-BPS	12–30	CF,BD	2
Flavobacterium-MC27	8.4		
Estuarine organisms			
Pseudomonas putida-TBT6	20.6	CF,AM,BD,O,LR	0
Enterobacter-TBT4	1683	CF,AM,BD,O,LR	4
Serratia-GIL1	106.7	CF,AM,BD,O,LR	3
K. pneumoniae-SHC16	ND[c]	CF,AM,BD,O,LR	3
Enterobacter-SHC10	414.6	CF,AM,BD,LR	1
Enterobacter-SHC2	18.8	CF,AM,BD,O,LR	1
Enterobacter-SHC11	ND	CF,AM,BD,O,LR	0
Enterobacter-TBT2	22.9	CF,AM,BD,O,LR	0
Pseudomonas-TBT8	ND	CF,AM,BD,O,LR	0
P. fluorescens-SHC6	1.8	CF,AM,BD,O,LR	0

[a] From Wuertz et al. [37]. Determined on Luria-Bertani agar for freshwater isolates and on tryptone agar for estuarine isolates.

[b] CR, Crosa and Falkow [12]; BD, Birnboim and Doly [4]; AM, Anderson and McKay [1]; O, Olson [22]; LR, Lee and Rasheed [17].

[c] ND = not determined.

Media

Trypticase soy agar (TSA) and trypticase soy broth (TSB) (BBL, Cockeysville, MD, USA) containing TBT were prepared as described [36] as were Luria-Bertani (LB) broth and SOC medium [25].

Electroporation

Electroporation experiments were performed using *E. coli* WM1100 as the TBT-sensitive recipient and plasmids isolated from estuarine strains SHC-2, GIL-1, SHC-10, SHC-16, and TBT-4, all of which are more resistant to TBT than *E. coli* WM1100. Crude plasmid preparations were obtained using the plasmid screening method which yielded the greatest number of plasmids with the particular isolate as listed in Table 1.

Competent cells were prepared by diluting an overnight culture 100-fold into 500 ml LB broth and incubating it at 37°C with vigorous shaking until an A_{600} of 0.5–1.0 was achieved. The culture was chilled on ice for 15 min and cells were harvested by centrifugation at $4000 \times g$ for 15 min at 4°C. Pellets were washed twice in 500 ml distilled H_2O and suspended in approximately 20 ml of 10% glycerol, centrifuged again, and resuspended in a final volume of 2–3 ml 10% glycerol at a cell concentration of approximately 10^{10} cells ml^{-1}. Cells were frozen on dry ice/ethanol in aliquots of 100 μl and stored at –90°C for up to six months. They were thawed at room temperature just prior to use and washed five times in 0.7 ml of 20% glycerol. At each washing step cells were resuspended by gentle repeated pipetting. After the last wash cells were resuspended in the fluid which remained once the supernatant layer had been poured off, leaving a dense slurry.

The electroporation apparatus was built as described by Speyer [28] and generously loaned by M.P. Shiaris and C. O'Rorke, University of Massachusetts, Boston, MA, USA. The transformation procedure was modified according to O'Rorke [23]. Approximately 10 μl of the plasmid preparation (not diluted or diluted in distilled water) was mixed with 30 μl of competent *E. coli* WM100 cells by vigorous vortexing for 60 s and stored on ice for up to 30 min. Transformation was performed in a cold room at 4°C. Twenty-five microliters of the mixture were applied to the lower of two metal plates of the electroporation apparatus, both plates having been disinfected with 70% ethanol. The upper plate (anode) was placed on top, pressed firmly with a lead weight, and the voltage was set at 300 V. After the capacitor was discharged, cells adhering to the top plate were washed into a sterile petri dish with a stream of SOC which had been warmed to 37°C. The cell suspension was immediately removed from the cold room and allowed to recover for at least 1 h at 37°C without shaking before plating the cells on tryptone agar containing TBT.

Plasmid curing

Overnight cultures in LB broth or TSB were diluted in saline and approximately 10^2–10^3 cells were inoculated into tubes of the same medium containing a range of concentrations of a filter-sterilized curing agent: acridine orange, ethidium bromide, novobiocin, or sodium dodecylsulfate (SDS). Cultures were grown until slightly turbid, then plated on tryptone or TSA plates. They were later replica-plated onto the same medium containing varying concentrations of TBT and, after suitable incubation, examined for colonies that had lost the ability to grow on the TBT-containing medium. For experiments involving elevated temperature, cultures were grown at slightly inhibitory temperatures (either 30°C or 37°C) in medium without TBT to an A_{600} of ~0.2, then plated, replicated and examined in a similar fashion.

Conjugation experiments

P. aeruginosa PAO1 containing pUM505 and *Beijerinckia* sp. MC-27 were mated by a modification of the 'combined conjugation approach' of Walter et al. [34]. The broth mating procedure was modified to increase the number of *Beijerinckia* cells recovered from the mating mixture. One milliliter of overnight *Beijerinckia* culture and 1 ml of overnight *P. aeruginosa* PAO1-(pUM505) culture in TSB were inoculated into 5 ml of TSB and incubated statically at 25°C for 24 h. The viscous pellicle which formed over the surface of the medium was removed and transferred to a tube containing 1 ml of 10 mM Tris-HCl tris(hydroxymethyl)-aminomethane buffer (pH 7.5) containing two or three 2-mm glass beads, and the tube was shaken by vortexing it for 30 s to break up the pellicle. Appropriate dilutions were plated on TSA containing 840 μM TBT, the plates were incubated for 24 h, and then examined for growth of the distinctive wrinkled *Beijerinckia* sp. MC-27 colonies. Plasmids were extracted from *P. aeruginosa* PAO1 colonies and *Beijerinckia* transconjugants by the method of Crosa and Falkow [12].

Growth curves

One milliliter of an overnight culture in TSB was used as inoculum for 100-ml volumes of TSB or TSB containing TBT in 500-ml sidearm flasks. TBT, dissolved in methanol, was added before the flasks were inoculated. Flasks without TBT received 1.0 ml of methanol. Flasks were incubated at 25°C with shaking and growth was followed using a Klett-Summerson photoelectric colorimeter equipped with a blue filter (Klett Manufacturing Company, New York, NY, USA), using sterile TSB-methanol without TBT as blank. Sidearms of flasks were calibrated. Klett units were converted to optical density as described by the manufacturer: OD = Klett units/500.

RESULTS AND DISCUSSION

Presence of plasmids

Six of 16 freshwater isolates and five of ten estuarine isolates contained plasmid DNA. Most of the plasmid-containing isolates contained more than one plasmid (Table 1). In all cases where pBluescript was included in the plasmid isolation preparation, the corresponding pBluescript band was evident in gels, indicating that the absence of nascent plasmids was not due to the action of endogenous nucleases in those strains. The possibility that plasmids may be present in very low copy number was investigated after extracting cells from 500-ml cultures of *Pseudomonas putida* TBT-6 and *Pseudomonas* sp. TBT-8 using the procedure of Anderson and McKay [1] as modified by Smith [27]. The DNA isolated was not susceptible

to restriction enzymes at this stage and was further purified by CsCl-ethidium bromide equilibrium density gradient centrifugation [25]. The absence of plasmids in these two strains was verified by the lack of a visual plasmid band in the gradient. We regard these strains as free of plasmids of the size reported to carry metal resistance genes (up to 240 kb). The techniques used may not have detected very large plasmids which might break up during the extraction procedure and band with chromosomal fragments during density-gradient centrifugation. The limit of detection was approximately 6 ng DNA per gel lane.

The TBT resistance of the organisms used varied by over a thousand-fold (Table 1). It is probable that TBT resistance need not be plasmid-mediated since several highly TBT-resistant, plasmid-free strains were identified. Moreover, Fukugawa and Suzuki [13] identified a chromosomal gene involved in resistance to TBT.

Plasmid curing

Strains containing plasmids were subjected to treatments known to cure other organisms of plasmids, and survivors were screened for progeny with increased sensitivity to TBT. Culturing cells at elevated temperatures did not yield derivatives with reduced TBT resistance, nor did treatment with any of four potential chemical curing agents. Those few colonies which did not grow after initial plating on TBT-containing agar plates were screened for loss of plasmids. All of these colonies proved to be TBT-resistant when they were checked again, and only one treatment (ethidium bromide for *Serratia* sp. strain Gil-1) resulted in the loss of a plasmid. This plasmid was small (4.9 kb) and could not be assigned a phenotype. It is possible that other plasmids were cured but these experiments were designed to detect cells which had lost TBT resistance rather than cells which had lost plasmids.

Electroporation

E. coli WM1100 was chosen as a cloning host for electroporation experiments because it was more sensitive to TBT than any of three other potential hosts examined: *E. coli* strains S171, HB101 and JM101; and because it was 5- to 470-fold more sensitive to TBT than any of the donor strains (data not shown). Five organisms were used as potential donors: *Enterobacter* spp. strains SHC-2, SHC-10 and TBT-4, *Klebsiella pneumoniae* strain SHC-16 and *Serratia* sp. strain Gil-1. Electroporation experiments did not produce any *E. coli* WM1100 colonies with increased TBT resistance. The transformation efficiency using the plasmids pBluescript or pBr322 was 3×10^6 μg^{-1} DNA, indicating that the method was effective for small plasmids. In several experiments some of the electroporated cells were immediately placed in TBT-containing outgrowth medium while others were plated on tryptone agar without TBT, incubated and cells were scraped from plates the following day, suspended in growth medium and plated on tryptone medium containing TBT. Occasionally a few isolated colonies were obtained. When such colonies were grown up and examined for plasmids, none was detected. These isolates are deemed to be spontaneous mutants which acquired TBT resistance via a chromosomal mutation, as described by Suzuki et al. [31].

Introduction of pUM505 into Beijerinckia *sp. MC-27*

Plasmid pUM505 from *P. aeruginosa* PAO1 was introduced into *Beijerinckia* sp. MC-27 at a frequency of 6×10^{-4}. TBT tolerance of the transconjugants on solid medium increased from 8.4 μM for *Beijerinckia* sp. MC-27 to 840 μM for *Beijerinckia* sp. MC-27-(pUM505). Of six TBT-resistant transconjugants examined for the presence of pUM505 by the method of Crosa and Falkow [12], each contained a corresponding plasmid band of the same size as pUM505 (Fig. 1). Moreover, the donor *P. aeruginosa* PAO1-(pUM505) was resistant to $\geqslant$ 1683 μM TBT and several spontaneous mutants which were resistant to only 1–10 μM TBT had lost the plasmid.

Growth curves of the recipient and the transconjugants indicate that TBT inhibited growth of the recipient *Beijerinckia* sp. MC-27 (Fig. 2(A)), but not of the transconjugant *Beijerinckia* sp. MC-27-(pUM505) (Fig. 2(B)). Plasmid transfer was detected only when the organisms were mated by the broth mating technique; the other methods of the 'combined conjugation approach', all of which employed solid media, did not yield transconjugants at a detectable level. Plasmid pUM505 was originally identified by Cervantes et al. [6] as a conjugative plasmid containing genes encoding chromium resistance [5]. The role of chromium resistance genes in TBT-resistant strains is not known. In fact, it is possible that chromosomal genes were transferred from *P. aeruginosa* PAO1-(pUM505)

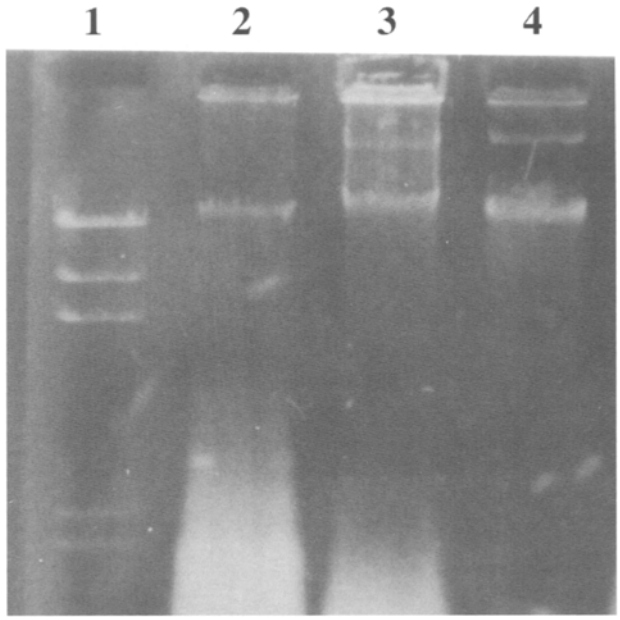

Fig. 1. Agarose gel (0.6%) electrophoresis of preparations from TBT-resistant *P. aeruginosa* PAO1-(pUM505), TBT-sensitive *Beijerinckia* sp. MC-27 and transconjugants. Lane 1, linear lambda DNA run as a check on effectiveness of the method, recognizing that it is linear and plasmid DNA is circular; lane 2, *Beijerinckia* sp. MC-27; lane 3, *P. aeruginosa* PAO1-(pUM505); lane 4, *Beijerinckia* sp. after mating with *P. aeruginosa*, from a colony selected on TBT-containing agar.

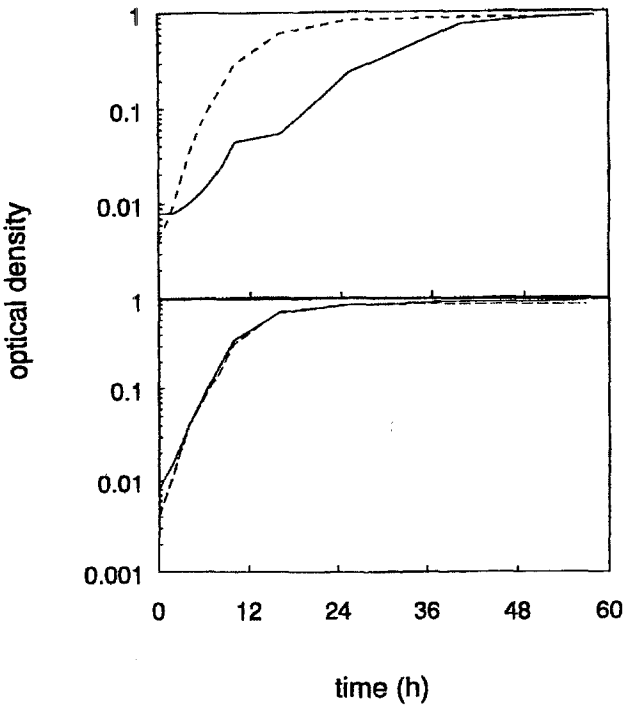

Fig. 2. Effect of TBT on growth of recipient and a transconjugant in TSB at 25°C with shaking. Top: solid line, *Beijerinckia* sp. MC-27 grown in the presence of 8.4 μM TBT; dashed line, *Beijerinckia* sp. MC-27-(pUM505) growing in TSB with 8.4 μM TBT. Bottom: same two organisms grown in the absence of TBT.

to *Beijerinckia* sp. MC-27 along with the plasmid, and that chromosomal genes play a role in increased TBT resistance in the MC-27 transconjugants. A large chromosomal gene involved in TBT resistance was cloned by Fukugawa and Suzuki [13] who suggested that TBT resistance is controlled by several genes.

When a transconjugant was subcultured at 24-h intervals in TSB without TBT, the numbers of TBT-resistant cells in the population decreased (Fig. 3). In the first culture there were

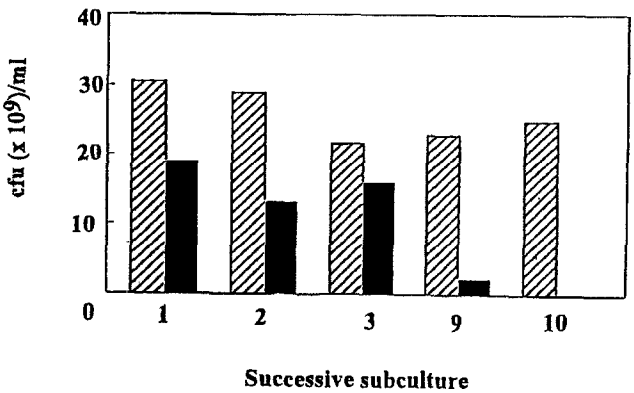

Fig. 3. Loss of TBT resistance during serial transfer on medium without TBT. A transconjugant was cultured in TSB at 25°C with shaking. At 24-h intervals it was transferred to fresh medium and a sample was taken, diluted and appropriate dilutions were plated on TSA with no added TBT and on TSA containing 840 μM TBT. Crosshatched bars, viable counts on TSA without TBT; solid bars, counts on TSA containing 840 μM TBT.

1.9×10^{10} resistant cells ml^{-1} and in the tenth serial culture, the number of resistant organisms was $<1 \times 10^9$ ml^{-1}. Selected colonies which grew up when the tenth culture was plated on medium without TBT did not yield plasmid DNA. These results are consistent with the suggestion that the plasmid is readily lost from the recipient *Beijerinckia* sp.

Among organometals only resistance to organomercurial compounds is known to be plasmid-mediated [20]. Resistances to inorganic forms of a number of heavy metals are plasmid-mediated, including Ag, As, Bi, Cd, Co, Cr, Cu, Hg, Ni, Te, Tl, Pb, and Zn [26]. We are unaware of any plasmids known to encode resistance to organotins or to inorganic tin. Belliveau et al. [3] isolated a *Pseudomonas cepacia* strain from polluted sediment which was resistant to 8.4 mM Sn and 4.8 mM Pb but did not recover any plasmids; and Summers and Jacoby [30] did not find a plasmid in a tin-resistant *P. aeruginosa*. Our results indicate that a plasmid can play a role in TBT resistance and perhaps in the transfer of TBT resistance between microorganisms. The mechanism(s) whereby bacteria can be resistant to tin compounds and the role of plasmids in the transfer of TBT resistance in situ are not yet known. This is the first report of involvement of plasmids in organotin resistance and we are optimistic of further applications of the finding.

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