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Reviews

Pathogenic Potential and Toxic Chemical Degradation by *Burkholderia cepacia*

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2,4,5-Trichlorophenoxyacetic acid (2,4,5-T) is a herbicide which is quite recalcitrant to microbial degradation. By using a highly selective chemostatic enrichment technique, we isolated a strain (AC1100) of *Burkholderia cepacia* that could utilize 2,4,5-T as its sole source of carbon and energy. The complete pathway of 2,4,5-T degradation by this strain has been delineated. This strain metabolizes 2,4,5-T via formation of 2,4,5-trichlorophenol (2,4,5-TCP), 5-chlorohydroxyquinol (5-CHQ), hydroxyquinol (HQ), maleylacetate (MA) and 3-oxoadipate. 3-Oxoadipate is finally metabolized by the chromosomally-encoded β-ketoadipate pathway. The genes for various steps of the 2,4,5-T degradative pathway have been cloned and sequenced. These genes are partially clustered as three sets on the chromosomes of *B. cepacia* AC1100. Pulsed-field gel electrophoresis analyses of whole chromosomes of this strain demonstrated that the genome is comprised of five replicons of 4.0, 2.7, 0.53, 0.34, and 0.15 mega base pairs (Mbp), designated replicons I to V respectively. The *tft* genes are located on smaller replicons: the *tftAB* cluster is on replicon IV, *tftEFGH* is on replicon III while copies of the *tftCD* genes are found on both replicons III and IV. Strain AC1100 is capable of removing large amounts of 2,4,5-T from 2,4,5-T-contaminated soil and restores the soil condition *B. cepacia* strains are also useful for application as biopesticides. Many strains of *B. cepacia* are, however, pathogenic and are often isolated from the lungs of cystic fibrosis patients or patients suffering from chronic granulomatous diseases. Thus there is great concern that release of *B. cepacia* in an open environment may greatly enhance and magnify the infection process. There is a need to assess the pathogenic potential of bioremediating organisms such as *B. cepacia*, which is briefly discussed in this article.

Key words: biodegradation, pathogen, virulence

Introduction

2,4,5-Trichlorophenoxyacetic acid (2,4,5-T), a major component of Agent Orange, has been widely released in the environment as a herbicide and defoliant, particularly during the Vietnam war. It is normally recalcitrant to microbial degradation. By using a strong chemostatic selection technique, we have isolated a strain of *Burkholderia cepacia*, designated AC1100 (formerly known as *Pseudomonas cepacia*) that can utilize 2,4,5-T as its sole source of carbon and energy (1). The strain AC1100 is known to remove more than 99% of 2,4,5-T from contaminated soil within a week when 2,4,5-T is present at 1000 μg/gm soil (2). Thus this strain, harboring the 2,4-D degradative plasmid pJP4, is capable of utilizing both 2,4-D (2,4-dichlorophenoxyacetic acid) and 2,4,5-T (which comprise Agent Orange) as its sole source of carbon and energy (3) and is considered a good candidate for the remediation of Agent Orange from the environment. Other strains of *B. cepacia* are known for their effectiveness in killing pathogenic soil fungi and nematodes, thereby promoting agricultural productivity (4) and have been proposed for environmental application. Unfortunately, many strains of *B. cepacia* have been isolated from patients suffering from cystic fibrosis (CF), chronic granulomatous disease and other diseases (5, 6). Since *B. cepacia* strains have high nutritional versatility and are capable of degrading many toxic chemicals, it is not clear if the strains isolated from the environment and considered suitable for environmental release, may demonstrate pathogenic potential similar to the strains isolated from infected patients. Thus an understanding of how the growth environment may influence the pathogenic potential of strains like *B. cepacia* AC1100 may provide significant insight on the role of environmental changes to the emergence of infectious organisms causing fatal infections in humans.

Results and Discussion

The 2,4,5-T degradative pathway and localization of the *tft* genes: The 2,4,5-T degradative pathway of *B. cepacia* AC1100, genes catalyzing various steps and the nature of the intermediates formed as well as the degradative enzymes have now been delineated (7, 8). In the initial steps, the *tftA* and *tftB* genes encode two subunits of the 2,4,5-T oxygenase which allows conversion of 2,4,5-T to 2,4,5-TCP (9). A two-component flavin-containing monooxygenase encoded by the *tftC* and *tftD* genes (10) catalyzes the *para*-hydroxylation of 2,4,5-TCP to yield 2,5-dichlorohydroquinone and then to 5-CHQ. The *tftG* gene product catalyzes dechlorination of 5-CHQ to yield 2-hydroxy-1, 4-benzoquinone, which is then reduced to HQ by the corresponding hydroxybenzoquinone reductase. HQ dioxygenase, encoded by the *tftH* gene, then catalyzes the ring cleavage to form MA which is reduced to 3-oxoadipate by the *tftE* gene product, MA reductase (8). 3-Oxoadipate is further metabolized to tricarboxylic acid cycle intermediates by the chromosomally - encoded β-ketoadipate pathway. The pathway of 2,4,5-T degradation, including the genes and intermediates of the pathway, is shown in Fig. 1.

The genomic organization of *B. cepacia* AC1100 has also been analyzed in order to better understand the evolution of 2,4,5-T biodegradative pathway and genomic plasticity. Using physical mapping techniques and pulsed-field gel electrophoresis, we have found that, unlike most bacteria, AC1100 possesses multiple chromosomes. The genome of AC1100 consists of five replicons of 4.0, 2.7, 0.53, 0.34 and 0.15 Mbp (10). It is unclear whether the smaller replicons should be classified as Fig. 2B (2) chromosomes or megaplasmids since mapping data show that *rrn* genes are confined to the two largest replicons. It is likely that AC1100 acquired its capability to metabolize 2,4,5-T by transfer of genes from other organisms present in a consortium during prolonged chemostatic selection. Several insertion sequence (IS) elements present in multiple copies in AC1100 may have played a central role in this process (7, 10). The unusual genomic structure and presence of IS elements may also provide mechanisms for the continued genomic recombination and rearrangements in this strain.

Three sets of cloned 2,4,5-T degradative genes, *tftAB, tftCD* and *tftEFGH* were mapped by Southern blot hybridization of whole chromosomes of wild type strain AC1100 separated by pulsed-field gel electrophoresis (10). The *tftAB* gene cluster, specifying the first step in the pathway, was located on the 0.34-Mbp replicon; however, the genes specifying the lower portion of the pathway, *tftEFGH*, were located on the 0.53-Mbp replicon (Fig. 2, A and B). Hybridization with the *tftCD* gene probe showed that the genes are duplicated and reside on both the 0.34 and 0.53-Mbp replicons, as well as other replicons (Fig. 2A). Using two primers corresponding to the N-terminal and an internal sequence of HBQ reductase (8), we have amplified a fragment as a probe. With the use of this probe, we have demonstrated the hybridization of the *hqr* gene with the 2.7-Mbp replicon (replicon II). This surprising distribution and the presence of adjacent IS elements suggest that the pathway was assembled in several independent events (10).

Determination of the pathogenic potential of *B. cepacia*

Since the clinical isolates of *B. cepacia* are successful colonizers of the CF lung (5, 6), an important question is their mode of evasion of host defense. We recently reported that mucoid *P. aeruginosa* strains, also successful

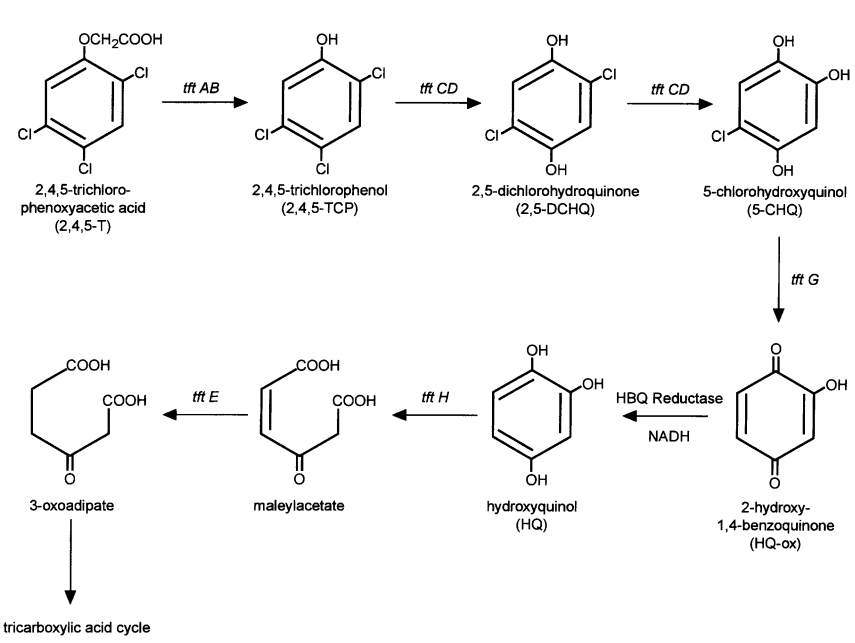

Fig. 1. Pathway of 2,4,5-T degradation. The various intermediates of the pathway are shown at the bottom while the genes are shown on top or side of the arrows. The gene for HBQ (hydroxybenzoquinone) reductase has not been characterized as yet.

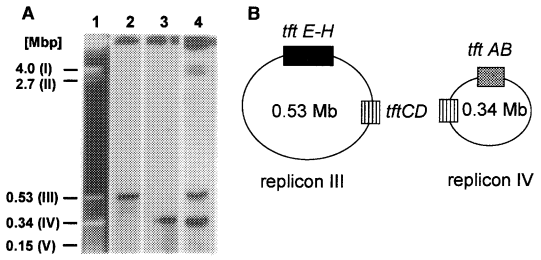

Fig. 2. Location of the *tft* genes in various replicons of *B. cepacia* strain AC1100. A: Lane 1 shows an ethidium bromide stained gel of the replicons separated by pulse-field gel electrophoresis. Lanes 2,3 and 4 are autoradiograms of the Southern blot hybridization of the same gel shown in lane 1 using different probes; lane 2, a 4.2 kb PstI-BamHI DNA fragment of the *tftEFGH* gene cluster as probe; lane 3, a 2.7 kb SstI-XbaI DNA fragment of the *tftAB* gene cluster as probe; lane 4, a 1 kb SstII-BamHI DNA fragment carrying *tftC* and part of *tftD* as probe. B: Diagrammatic representation of the three *tft* gene clusters in two *B. cepacia* AC1100 replicons.

colonizers of the CF lung, as well as *Vibrio cholerae*, an intestinal colonizer, evade the host defense by killing macrophages, the first line of defense in the host (11, 12). We demonstrated (11) that mucoid *P. aeruginosa*, but not a nonmucoid laboratory strain PAO1, secretes various ATP-utilizing enzymes that modify ATP. The modified ATP appears to be strong agonists of some macrophage surface-associated receptors called the purinergic or P2 receptors (13). Among the P2 receptors is a group called the P2Z (or $P2X_7$) receptor which on activation in presence of mM concentrations of ATP allows pore formation on the membranes of macrophages through which low molecular weight (upto 900 dalton) solutes can pass through leading to macrophage cell death (14). Macrophages under certain circumstances efflux ATP in response to bacterial LPS (15) or intact bacteria (16), which allows P2Z receptor activation, causing macrophage cell death. Thus P2Z receptor activation and consequent cell death through efflux of mM concentration of ATP is an accepted process for macrophages since if the macrophage dies, the engulfed bacteria die with it. Successful pathogens, however, have figured out how macrophages undergo cell death through P2Z receptor activation, and accelerate the process by secreting enzymes that modify the ATP to various forms that are better activators of the P2Z and other receptors, thereby enhancing macrophage cell death (11). It was, therefore, of interest to us to determine if *B. cepacia* may employ a similar ploy to evade the host defense by killing macrophages through enhanced P2Z receptor activation. We found out that several CF isolate strains of *B. cepacia* and several environmental strains, including the 2,4,5-T degrading strain of *B. cepacia* AC1100, could secrete ATP-utilizing enzymes such as ATPase, adenylate kinase (Ak), 5'-nucleotidase and nucleoside diphosphate kinase (Ndk). In general, the environmental isolates secreted much less 5'-nucleotidase and Ak, but more Ndk, than the CF-isolates (strains 27 and 38). The secretion of these enzymes was significantly enhanced in presence of eukaryotic proteins such as human plasma α2-macroglobulin. When the cells of the CF-isolate strain 38 were incubated in presence of 300 μg/ml of α2-macroglobulin, secretion of the enzymes reached a steady state in about 45 to 60 minutes (17). Commensurate with the level of the secreted enzymes, the supernatant growth medium of the CF-isolate strains demonstrated a much higher macrophage killing activity in presence of ATP, than the supernatants derived from the environmental isolates (17). The ATP-inducible enhanced killing of the macrophages was inhibited on pretreatment of the macrophages with oxidized ATP (oATP). oATP is a known inhibitor of P2Z receptor activation, which binds the receptor irreversibly and blocks its subsequent activation by ATP. Thus the inhibition of enhanced macrophage killing by the supernatants of clinical strains, when the macrophages were pretreated with oATP, clearly suggested that P2Z receptor activation was important in the enhanced killing of the macrophages by the supernatants of CF isolates of *B. cepacia* (17). Since the environmental isolates showed reduced level of secretion of some of the ATP-utilizing enzymes (5'-nucleotidase, Ak, etc) and reduced cytotoxicity towards macrophages, it would be of great interest to examine if environmental isolates are inherently deficient in the production of cytotoxic agents or whether their potential to produce cytotoxic agents could be enhanced either by passage through host cells or during prolonged growth in presence of specific eukaryotic proteins such as α2-macroglobulin. It would also be important to purify the secreted ATP-utilizing enzymes to demonstrate their individual roles in the ATP-inducible macrophages cytotoxicity assays, *viz*, if there are specific enzymes that act on ATP to produce products that are better able to kill macrophages through a higher level of activation of P2Z or other purinergic receptors. Indeed, purified Ak was shown to behave as a virulence factor in *P. aeruginosa* since it allowed enhanced macrophage cell death in presence of its

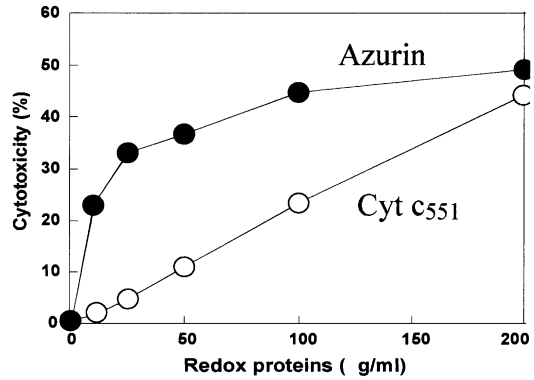

Fig. 3. Cytotoxicity of two purified redox proteins, azurin and cytochrome c_{551} (Cyt c_{551}), towards macrophages. The cytotoxicity determination was done as described in reference 22.

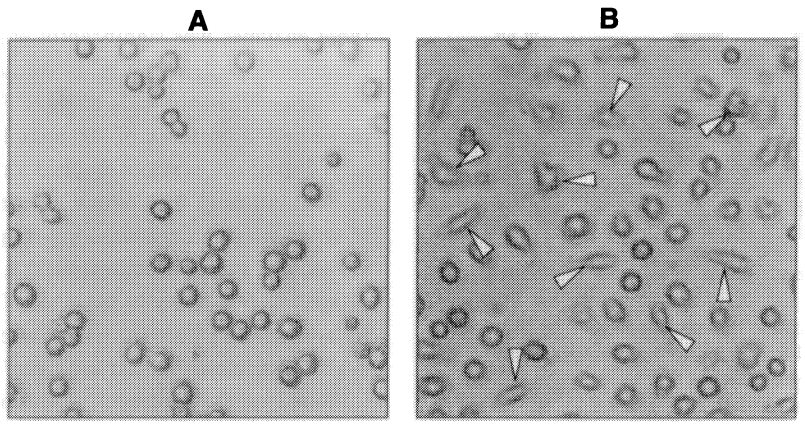

Fig. 4. Changes in morphology of macrophages treated with (B) or without (A) azurin (100 μg/ml) for 18 hours. The various morphological forms in B are shown with arrowheads.

substrates ADP or ATP+AMP (18). Once the enzymes are purified, their genes can be cloned and inactivated by cassette mutagenesis. The ability of *B. cepacia* clinical strains, having knock-out mutations in the genes encoding 5′-nucleotidase, Ak, Ndk, etc, to secrete cytotoxic factors for P2Z receptor-mediated macrophage killing, will demonstrate if one or more of these secreted enzymes may play a critical role in *B. cepacia* virulence.

In addition to the ATP-utilizing enzymes secreted by *P. aeruginosa* (11) or *B. cepacia* (17), we have recently reported the secretion from *P. aeruginosa* of another set of enzymes such as azurin and cytochrome c_{551} that are involved in electron transfer reactions in the cell (19). These redox enzymes were shown to induce apoptosis in macrophages through activation of the caspase cascade (19). Similar azurin and cytochrome c_{551} homologues are also produced by *B. cepacia*. Azurin is a blue copper containing protein while cytochrome c_{551} is a haem-containing protein involved in electron transfer during denitrification, and a great deal is known about their secondary structures and the role of critical amino acids in the process (20, 21). In order to determine if laboratory-purified azurin and cytochrome c_{551} might demonstrate cytotoxicity towards macrophages, we hyperexpressed cloned azurin and cytochrome c_{551} genes in *Escherichia coli*, purified the proteins by column chromatography, and determined their cytotoxicity by the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (22). This assay determines the number of live cells during a treatment so that compared to untreated control with 100% cell population, the extent of killed cells, expressed as percent cytotoxicity, can be determined. The results of such an assay is shown in Fig. 3. Purified azurin at a concentration of 50 to 100 μg/ml demonstrated substantial cytotoxicity to the macrophages. Cytochrome c_{551} had similar effect, *albeit* at a higher concentration (Fig. 3). Phase contrast microscopy demonstrated altered morphology of the treated macrophages. While buffer-treated macrophages had more or less rounded, compact shapes, many of the macrophages treated with azurin showed cell swelling, membrane blebbing and vacuolization, demonstrating extensive apoptosis (Fig. 4), as reported earlier (19). Thus these redox proteins represent potential virulence factors elaborated by *P. aeruginosa* and *B. cepacia*.

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