An efficient cyanide-degrading *Bacillus pumilus* strain

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A Gram-positive, aerobic, endospore-forming bacterium was isolated by an enrichment technique for the ability to degrade cyanide and was identified as a *Bacillus pumilus* strain. The bacterium rapidly degraded 100 mg l⁻¹ of free cyanide in the absence of added inorganic and organic substances. The ability to degrade cyanide was linked to the growth phase and was not exhibited before late exponential/early stationary phase. Cyanide-degrading activity could not be induced before this time by the addition of 20 mg cyanide l⁻¹. Production of the cyanide-degrading activity required 0.01 mg Mn²⁺ l⁻¹ and did not occur at Mn²⁺ concentrations below 0.002 mg l⁻¹. Cyanide-degrading activity was intracellular and cell-free extracts rapidly degraded cyanide.

Introduction

The current global concern with environmental problems has made the biological detoxification of cyanide an attractive alternative to chemical detoxification procedures. Biological detoxification methods may be both cheaper and more environmentally acceptable than chemical methods. Most attempts to develop biological processes for the detoxification of cyanide-containing effluents from industry have concentrated on cyanide-degrading fungi (Knowles & Bunch, 1986). ICI Bioproducts markets a product called Cyclear for this purpose (ICI Bioproducts Patent Application, 1987). This is a preparation of cyanide hydratase from a strain of *Fusarium lateritium*.

However, relatively little work appears to have been done on the characterization and development of bacterial cell-free extracts or enzymes able to degrade cyanide (although Novo-Nordisk A/S has recently filed a patent for the decomposition of metal-cyano complexes using enzymes from a strain of the bacterium *Alcaligenes*; Novo-Nordisk A/S Patent Application, 1987). The Homestake Mine in Lead, South Dakota, USA, utilizes a biological treatment plant for the detoxification of cyanide-containing wastewaters (Whitlock & Mudder, 1986). The process involves initial oxidation of free and metal cyanides, as well as thiocyanate, to ammonia and carbonate by a strain of *Pseudomonas paucimobilis* followed by the oxidation of the ammonia to nitrite and then nitrate by strains of *Nitrosomonas* and *Nitrobacter*.

Harris & Knowles (1983a, b) and Rollinson *et al.* (1987) reported the utilization of cyanide as a source of nitrogen by *Pseudomonas fluorescens* NCIMB 11764. Dorr & Knowles (1989) described cyanide oxygenase and cyanase activities in this organism. White *et al.* (1988) reported the conversion of cyanide to formate and ammonia by a pseudomonad isolated from industrial wastewater. Skowronski & Strobel (1969) isolated a strain of *Bacillus pumilus* which could survive in solutions of KCN of up to 2.5 M and grew in media containing 0.1 M-KCN (2600 mg CN⁻ l⁻¹). Both living and dead cells decreased the cyanide concentration of this medium, but the dead cells did so to a much smaller extent. The cyanide was degraded to carbon dioxide and ammonia. Castric & Strobel (1969) and Castric & Conn (1971) reported the metabolism of cyanide by a growing culture of *Bacillus megaterium* and proposed the presence of a metabolic pathway involving the condensation of serine with cyanide to form beta-cyanoalanine followed by the hydrolysis of the latter to asparagine and then to aspartic acid. McFeters *et al.* (1970) studied the effect of cyanide on the respiration and cytochrome content of a highly cyanide-resistant strain of *Bacillus cereus*. These three *Bacillus* species were all isolated from the same site, viz. Fargo clay collected from a field near Fargo, North Dakota, USA, which had been cropped in flax for 73 consecutive years (Skowronski & Strobel, 1969).

We have embarked on a project to assess the potential for the rapid bacterial detoxification of cyanide-containing effluents generated by the mining industry (approx-

mately 100 mg CN⁻ l⁻¹). We report the isolation and characterization of an efficient cyanide-degrading *Bacillus pumilus* strain.

Methods

Selection of cyanide-degrading micro-organisms. A continuous selection system was assembled using a stoppered round-bottomed flask into which a small sample of soil material from a cyanide waste water (slimes) dam from a mining company in the Transvaal, South Africa, had been placed. The system was operated at 25 °C. Separate feed solutions containing 10 mg CN⁻ l⁻¹ (as NaCN) and tapwater (to supply trace elements) were dripped into the flask at a dilution rate of 0.05 h⁻¹. The pH was not controlled. Filtered air was bubbled through the solution in the flask to aerate it. The overflow from the flask was passed over activated carbon (to trap any micro-organisms washed out of the flask) before passing into a waste drum. The cyanide concentration in the cyanide-containing influent was increased incrementally over 2 months to a final concentration of 100 mg CN⁻ l⁻¹.

Samples were removed from the flask from time to time and plated onto nutrient agar. A circular piece (diameter 8 cm) of Whatman's no. 1 filter paper was placed in the lid of the Petri dish and moistened with 1.5 ml of a 100 mg l⁻¹ solution of NaCN (pH 10.5). The plates were sealed with Parafilm and incubated at 30 °C. Bacterial colonies were subcultured on the sealed nutrient agar plates with the cyanide filter paper discs to obtain pure colonies. To test for cyanide degradation, isolated colonies growing on the sealed nutrient agar plates were resuspended in 3 ml sterile distilled water and inoculated into 200 ml screwcap glass bottles containing 100 ml sterile distilled water and 50 mg CN⁻ l⁻¹ (pH 10.4). The bottles were tightly sealed with screw caps containing rubber seals and incubated at 30 °C for 14 d. Samples were removed at 24 h intervals and assayed for residual cyanide. In subsequent experiments with selected strains, 200 ml nutrient broth cultures (pH 7.4) grown at 30 °C with shaking for 14 h were harvested by centrifugation and resuspended in 100 ml sterile distilled water and transferred to screwcap bottles. Cyanide (as KCN) was added to the cell suspension to a final concentration of 100 mg CN⁻ l⁻¹ (final pH 10.5). The bottles were incubated at 30 °C and assayed for remaining cyanide at half-hourly or hourly intervals.

Determination of residual cyanide. Cyanide-containing solutions were assayed by a modification of the picric acid method of Fisher & Brown (1952). A linear calibration curve was obtained with the standard cyanide solutions used in the assays. Control experiments involving correlation with a gas chromatography assay indicated that the picric acid test was suitable and reliable. Cyanide-containing solutions (0.05 ml) were added to 0.1 ml of a solution containing 0.5% (w/v) picric acid and 0.25 M-Na₂CO₃. The resulting solutions were placed in a boiling waterbath for 5 min, diluted to 1 ml with 0.85 ml distilled water, and cooled in tapwater for 30 min before reading the absorbance at 520 nm against a blank of distilled water and the picric acid/Na₂CO₃ solution. The absorbance of the cyanide-picric acid reaction product was stable for many hours (Fisher & Brown, 1952).

Characterization of cyanide-degrading bacteria. Forty-eight bacterial isolates were tested for the ability to degrade free cyanide. The 12 best strains were chosen for further study and characterized with respect to Gram stain, catalase and oxidase reactions, and ability to grow anaerobically. Further characterization was carried out using classification tests described by Gordon (1973), Cruickshank *et al.* (1975), Norris *et al.* (1981) and Sneath (1986).

Preparation and assay of cell-free extracts. Nutrient broth cultures (200 ml) grown at 30 °C with shaking for 14 h were harvested by centrifugation at 10000 g for 10 min. Cells were washed twice with

20 ml ice-cold 50 mM-potassium phosphate/50 mM-NaCl buffer, pH 7.4. The cells were finally resuspended in 10 ml ice-cold buffer and disrupted by two passages through an Aminco French pressure cell at 1.1 × 10⁵ kPa. The disrupted cell suspension was centrifuged at 27000 g for 30 min at 4 °C. The supernatant was retained and a 2 ml sample, diluted 1 in 20 in 50 mM-potassium phosphate/50 mM-NaCl buffer (pH 7.4), was prewarmed at 30 °C for 1 h. Cyanide was added to give a final concentration of 102 mg l⁻¹ and triplicate samples (0.05 ml) were assayed for residual cyanide after 0, 15, 30, 60, 120 and 240 min. Protein concentrations in cell-free extracts were determined by the Lowry method.

Results and Discussion

Isolation and characterization of cyanide-degrading bacteria

All 12 of the bacterial strains selected for further study were slender, aerobic, Gram-positive, endospore-forming rods. These characteristics are indicative of the genus *Bacillus*. Spores were located centrally. No vacuolation was visible in cells grown on glucose agar. All 12 strains were unable to hydrolyse starch or reduce nitrate, characteristics which suggested that the organisms were strains of *B. pumilus* (Knight & Proom, 1950). Furthermore, all 12 strains exhibited an absolute requirement for biotin for growth on minimal medium. This was taken as confirmation that the 12 isolates were *B. pumilus* strains.

All 12 isolates grew poorly in aerobic overnight cultures of Difco nutrient broth and cells from such cultures did not degrade cyanide. The same strains grew well under the same conditions in Oxoid nutrient broth and rapidly degraded cyanide (Fig. 1). Studies with

Fig. 1. Cyanide degradation by cells of *B. pumilus* C1. Cells grown in 200 ml Oxoid nutrient broth at 30 °C with shaking for 14 h were harvested by centrifugation and resuspended in screwcap bottles containing 100 ml sterile distilled water and 100 mg CN⁻ l⁻¹ (pH 10.5). The suspension was incubated at 30 °C without shaking. The cyanide concentration was assayed at 30 min intervals for 2 h. After 2 h an additional 100 mg CN⁻ l⁻¹ was added and the cyanide concentration determined for a further 2 h. O, Incubation with *B. pumilus* C1 cells; ■, control (cyanide + water). The SEM values were 5–10% of the values in the Figure (four determinations were done).

Fig. 2. Relationship between phase of growth and cyanide degradation by cells of *B. pumilus* C1. Cultures (200 ml) were grown aerobically in Oxoid nutrient broth (a) and cell samples were assayed for cyanide degradation after 2 h (lag phase, Δ), 5.5 h (mid-exponential phase, $\diamond$) and 12.75 h (stationary phase, $\square$) (b). The cultures were harvested by centrifugation and resuspended in screwcap bottles containing 100 ml sterile distilled water and 100 mg CN⁻ l⁻¹ (pH 10.5). The suspensions were incubated at 30 °C without shaking and the cyanide concentration was assayed at 30 min intervals for 1.5 h. The SEM values were 5–10% of the values in the Figure (three determinations were done).

mixtures of Oxoid and Difco nutrient broths indicated that the Difco nutrient broth exerted an inhibitory effect on the aerobic growth and cyanide-degrading ability of these isolates (results not shown). All 12 strains were unable to grow aerobically on minimal agar plates with KCN as the only nitrogen source using the filter-paper method described by Harris & Knowles (1983a). The strain which displayed the greatest ability to degrade cyanide was chosen for more detailed study and was designated *B. pumilus* C1.

Degradation of cyanide by *B. pumilus* C1

B. pumilus C1 degraded free cyanide (100 mg l⁻¹, supplied as KCN) very efficiently (Fig. 1). The free cyanide was degraded within 2 h and following the further addition of 100 mg cyanide l⁻¹ the cells continued to degrade the cyanide, albeit at a slower rate. Cells of *B. pumilus* C1 cultivated in Oxoid nutrient broth and harvested during the lag or exponential phase were unable to degrade cyanide, whereas late exponential/early stationary phase cells were able to do so (Fig. 2). The addition of 20 mg cyanide l⁻¹ to the medium did not induce the cyanide-degrading activity in lag- or exponential-phase cells.

Fig. 3. Effect of Mn²⁺ in the growth medium on cyanide degradation by cells of *B. pumilus* C1. Cells were grown at 30 °C with shaking for 14 h in Oxoid nutrient broth (20 ml) containing increasing concentrations of Mn²⁺. Cultures were harvested by centrifugation and resuspended in standard containers containing 10 ml sterile distilled water and 100 mg CN⁻ l⁻¹ without added Mn²⁺ (pH 10.5). The suspensions were incubated at 30 °C without shaking and the cyanide concentrations were assayed at 30 min intervals for 1.5 h. Mn²⁺ concentration (mg l⁻¹) in the growth medium: 0 (∇); 0.002 ($\circ$); 0.01 ($\square$); 0.05 ($\diamond$). Δ , Control (cyanide + water). The SEM values were 5–10% of the values in the Figure (three determinations were done).

B. pumilus cells grown in Oxoid nutrient broth made with Millipore deionized water were unable to degrade cyanide, whereas cells grown in Oxoid nutrient broth made with tap water rapidly degraded cyanide. An investigation of the elements present in the tap water showed that manganese was specifically required in the growth medium for the production of the cyanide-degrading activity of the *B. pumilus* cells. The addition of 0.01 mg Mn²⁺ l⁻¹ to Oxoid nutrient broth made with Millipore deionized water resulted in the production of cyanide-degrading activity (Fig. 3). The cyanide-degrading activity was not produced when cells were grown at Mn²⁺ concentrations below 0.002 mg l⁻¹. The presence or absence in the growth medium of the other elements shown to be present in tap water did not affect the cyanide-degrading ability of the *B. pumilus* cells (results not shown). Manganese may be required either for induction of the gene(s) controlling cyanide-degrading enzyme activity, or as a component of the cyanide-degrading enzyme activity.

Cell-free extracts were diluted to a concentration of 0.2 mg protein ml⁻¹ and assayed for their ability to degrade cyanide. The cell-free extracts degraded cyanide efficiently and the specific activity for cyanide degradation was 0.28 μ mol CN⁻ used min⁻¹ (mg protein)⁻¹. The cyanide-degrading activity of the cell-free extracts appeared to be stable and could be stored at 4 °C or -20 °C without loss of activity. Degradation of cyanide by cell-free culture supernatants was not detected.

Since *B. pumilus* C1 yielded crude cell extracts which

were relatively stable and degraded cyanide efficiently after storage, this strain has potential in the development of a cell-free biological treatment system. The absolute requirement for Mn^{2+} for the production of the cyanide-degrading activity appears to be a specific characteristic of *B. pumilus* C1.

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