

Toxin production by *Bacillus pumilus*

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Abstract

Two strains of *Bacillus pumilus* (M11 and M38) and one strain each of *Bacillus cereus* (M27), *Bacillus subtilis* (M67), and *Enterobacter agglomerans* (M14) were identified from the air of Lancashire cotton mills. These strains were tested for cytopathic effects in Vero cells; *B pumilus* and *B cereus* strains were also examined for haemolytic activity, lecithinase production, and proteolytic action on casein. Rounding and clumping of the Vero cells occurred after the addition of supernatants prepared from *B pumilus* and *B cereus* strains; finger-like projections developed in the cells treated with *B pumilus* supernatants. Minimal effects occurred with *B subtilis* and *E agglomerans*. After two hours of exposure *B pumilus* (M11) produced the greatest effect, but treatment with trypan blue showed that most cells exposed to the M11 strain were still alive after 96 hours of exposure; those exposed to the supernatant prepared from the M38 strain of *B pumilus* were dead. Sheep erythrocytes were lysed more rapidly by *B cereus* than by *B pumilus*, *B cereus* (strongly positive) had a greater effect on lecithin than either of the *B pumilus* strains (M38 weakly positive, M11 negative). All hydrolysed casein but the effect was more rapid with one of the *B pumilus* (M11) strains.

It is concluded that not only do the toxins of *B pumilus* differ from those of *B cereus*, but there are also differences between the toxins produced by the two strains of *B pumilus* (M11 and M38).

In recent years attention has focused on the role of bacterial toxins in causing disease. Flindt has shown that preparations from *Bacillus subtilis* were responsible for causing respiratory symptoms in workers producing washing powders containing enzymes.¹ *B pumilus*, which produces toxin, has been isolated from guinea-pigs with experimentally induced enterocolitis associated with clindamycin² and toxin from *B cereus* has been shown to cause food poisoning.³

Current opinion is that endotoxin from *Enterobacter agglomerans* (a Gram negative bacterium found on plants) has an important role in producing the symptoms of byssinosis, the cotton and flax workers' respiratory disease.⁴ When the bacterial contents of the air in Lancashire cotton mills were studied by

Tuffnell,⁵ however he did not find *Ent agglomerans* (previously known as *Erwinia herbicola*), although his culture media would have supported the growth of these bacteria. He reported that the predominant species were *B pumilus* and *B subtilis* in the cotton mills where byssinosis occurred and *B megaterium* in the jute mills, where the disease was absent. Twenty five years later Tuxford and Moogan isolated these three species of *Bacillus* and also *Ent agglomerans* among 21 different airborne bacterial species in the Lancashire cotton mills (the jute mills had closed).⁶ It seemed logical, therefore, to examine airborne bacteria, including *B pumilus* and *Ent agglomerans* isolated from the Lancashire cotton mills, for toxin production.

There are various techniques for detecting toxins produced by bacteria including observation of the cytopathic effects on living cells. Monolayers of Vero cells (derived from green monkey kidney) have been used for the detection of *Clostridium perfringens* toxin,⁷ *B cereus* toxin,⁸ and enterotoxin of enteropathic *Escherichia coli*.⁹ As this cell line has been used to detect toxins from both Gram negative and Gram positive bacteria, including *B cereus*, it seemed to be suitable for testing both *Bacillus* species and *Ent agglomerans*.

Other manifestations of toxin production by the *Bacillus* species are the ability to lyse erythrocytes of different animal species (haemolysins), to attack proteins (proteolytic activity), and to break down lecithin by the action of lecithinase. Some of these properties have been used by Turnbull and Kramer¹⁰ in their scheme for the identification of *B cereus*, and it was decided to modify their techniques to detect similar properties in strains of *B pumilus* and some of the other species.

Methods

BACTERIA

The bacteria used in this series of experiments were from a collection isolated in 1986 from the air of 18 Lancashire cotton spinning mills, a representative number of which were stored on nutrient agar slopes for future use. Included in the collection were 14 strains of *B pumilus*, four of *B cereus*, two of *Ent agglomerans* and five strains of *B subtilis*. Fifty six strains from 21 bacterial species were screened initially for toxic effects on Vero cells; 12 out of the 14 strains of *B pumilus* and three out of four of *B cereus* were cytopathic; all the *B subtilis* and *Ent agglomerans* strains proved to be non-cytopathic. Two strains of *B pumilus* (M11 and M38) and one of *B cereus* (M27) were selected

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for further examination. Strains of *B subtilis* (M67) and *Ent agglomerans* (M14) were also included as non-cytopathic controls in some of the experiments.

The identities of the previously isolated strains were reconfirmed by Gram staining, cultural appearances, and API galleries for biochemical characterisation (20B for *Bacillus* species and 20E for *Ent agglomerans*). Cultures were also tested for their ability to grow anaerobically, and spore stains were performed on older cultures of *Bacillus* species.

PREPARATION OF SUPERNATANTS FOR INOCULATION INTO TISSUE CULTURE

The bacteria were cultured overnight in nutrient broth at 37°C, nutrient agar (Oxoid Broth No 2 with 1% agar) bijoux slopes were inoculated from these cultures and incubated for 48 hours at 37°C. The cultures were harvested into 0.5 ml of Gibco's minimal essential medium (MEM), supplemented with 1% glutamine and 1% fetal calf serum. The suspensions were mixed for 30 seconds on a vortex mixer, left at room temperature for 60 minutes, then centrifuged for 15 minutes at 1800 × *g*. Before use the supernatants were filtered through a sterile millipore filter of 0.2 µm pore size. Uninoculated nutrient agar slopes were treated in the same way to provide bacteria-free supernatants to act as negative controls.

TISSUE CULTURE

Wells of tissue culture microtitre plates (Falcon) were inoculated with 200 µl of Vero cells grown in MEM supplemented with 10% fetal calf serum, and incubated at 37°C for 24 to 48 hours in a carbon dioxide incubator until the cell sheet was confluent. The growth medium was then replaced with 200 µl of bacterial supernatant and duplicate plates were incubated at 37°C in 5% carbon dioxide and examined at intervals (30 and 60 minutes, two, four, 24, and 48 hours) using an inverted microscope to detect cytopathic effects. Supernatants were tested in duplicate and supernatants from non-cytopathic species, similarly treated, were included as negative controls. To establish whether the effect was cytotoxic (more than 50% of cells showing rounding) or cytotoxic (death of more than 50% of cells),¹¹ trypan blue was added to several wells each day to determine the number of dead cells. In a separate experiment the regenerative capacity of the tissue culture was tested by removing supernatants after 30 and 60 minutes of exposure and replacing with 200 µl of either growth or maintenance medium. The plates were reincubated at 37°C in carbon dioxide and the Vero cells were examined daily.

OTHER TOXIC EFFECTS

Two strains of *B pumilus* (M11 and M38) and the M27 strain of *B cereus* (as a positive control) were examined for haemolytic, phospholipolytic, and proteolytic effects using the methods of Turnbull and Kramer,¹⁰ modified as follows: Supernatants were prepared by inoculating one drop of an 18 hour nutrient broth culture (Oxoid) into 50 ml of brain heart infusion broth (Oxoid) and incubating in a shaking water bath at 37°C for 48 hours. Bacteria were removed by centrifugation and filtration. Removal was confirmed by inoculating the filtrates onto nutrient agar plates, incubating overnight at 37°C and examining for absence of bacterial growth.

Haemolytic effect

Twofold dilutions of the supernatants in phosphate buffered saline (PBS) (Dulbecco A tablets; Oxoid) were made in WHO plates and equal volumes of 0.5% washed sheep erythrocytes suspended in PBS were added to give final concentrations in the wells from 1 in 10 to 1 in 1240. The plates were incubated at 37°C and inspected for haemolysis after four and 24 hours.

Phospholipolytic activity

This was determined by placing 50 µl of supernatant in 5 mm diameter wells cut in lecithin agar (10% egg yolk emulsion agar, Oxoid). The plates were incubated at 37°C and the level of activity determined by measuring the diameter of the zones of opacity around the wells after four, eight, and 24 hours' incubation.

Proteolytic activity

Wells of 5 mm diameter were cut in casein (50% skimmed milk) agar plates; these were filled with 50 µl of supernatant and incubated at 37°C. Zone diameters around the wells were measured after four, eight, and 24 hours.

All experiments were performed in duplicate and were repeated.

Results

Typical toxic effects on the Vero cell sheets are shown in the figs 1–4. In the wells containing supernatant with cytopathic properties the cells showed rounding and clumping with the formation of holes in the cell sheet; long projections developed in cells treated with *B pumilus* supernatants (fig 1). *B cereus* caused a cytopathic effect but without the finger-like projections (fig 2); *B subtilis* supernatant had little or no effect on the Vero cells, and minimal cytopathic effects were also observed in those wells treated with *Ent agglomerans* (fig 3).

Effects of *Bacillus* supernatants

Bacterial species	Sheep cell haemolysin		Lecithinase		Protease	
	Hours	Titre	Hours	Zone	Hours	Zone
<i>B cereus</i> M27	4	1/80	24	20 mm	24	20 mm
<i>B pumilus</i> M11	24	1/40	24	0 mm	8	20 mm
<i>B pumilus</i> M38	24	1/40	24	7 mm	24	20 mm

Figure 1 *B pumilus* (M11): cytopathic effect on Vero cells after 18 hours' incubation.

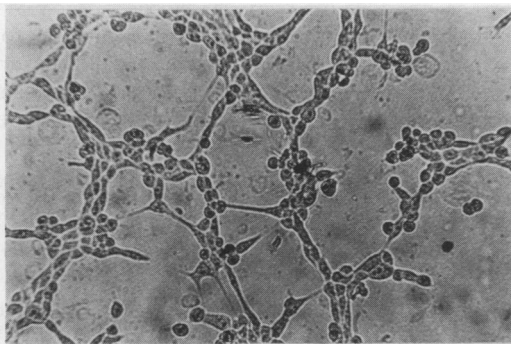


Figure 2 *B cereus*: cytopathic effect on Vero cells after 18 hours' incubation.

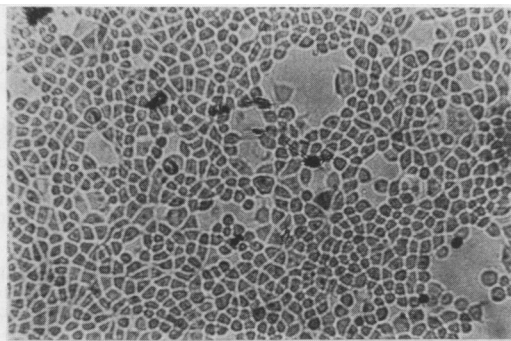


Figure 3 *E agglomerans*: minimal cytopathic effect on Vero cells after 48 hours' incubation.

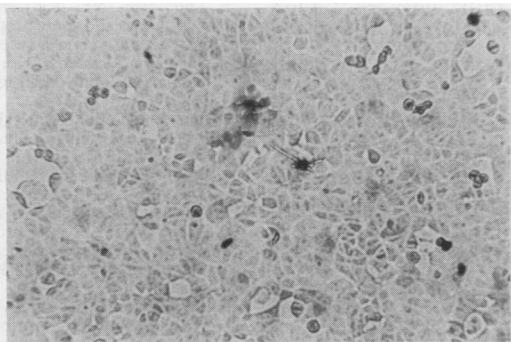
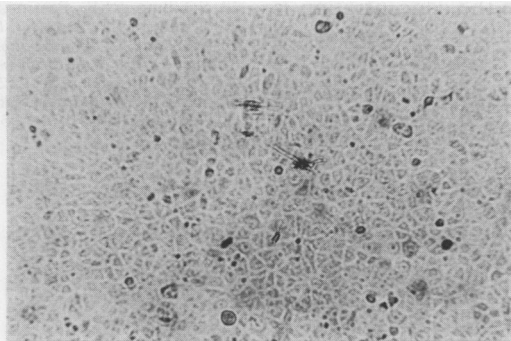


Figure 4 Vero cells incubated without bacterial supernatant for 48 hours.



Negative control supernatants had no effect on the tissue culture (fig 4). *B pumilus* (M11) and *B cereus* produced maximum effects on the cell sheet within two hours of inoculation; *B pumilus* (M38) had little effect in the first 24 hours, but by 48 hours showed a typical *B pumilus* type effect on the cell sheet (fig 1). Treatment with trypan blue showed that at 48 hours very few cells treated with *B pumilus* (M38) had died, but by 96 hours most were dead; this was considered to be a cytotoxic effect.¹¹ Examination of the cells exposed to the M11 strain of *B pumilus* showed a cytotoxic effect¹¹ in that most cells were still alive after 96 hours. Immediately after the development of

the first holes in the cell sheet the supernatant was replaced with either maintenance medium or growth medium and the plates were re-incubated for 48 hours. Cell sheets treated with maintenance medium were unchanged but regeneration of the cell sheet had occurred in those wells containing growth medium and the cytopathic effect was no longer visible.

Results of the other effects of the supernatants of *B cereus* and *B pumilus* are shown in the table. Sheep erythrocytes were lysed in four hours by *B cereus* supernatant at a titre of 1/80; the *B pumilus* supernatants at a titre of 1/40 produced haemolysis in 24 hours. Zone sites of 20 mm in the lecithin plates were observed after 24 hours' incubation with *B cereus*. Different effects were observed with the two strains of *B pumilus*, M38 produced very small zones (7 mm diameter) after 24 hours' incubation, whereas M11 had no effect on the lecithin even after prolonged incubation. Zones of 20 mm in the casein plates were produced by all three strains, but the time to produce the zones varied; after eight hours a zone had occurred around M11; it took 24 hours' incubation to develop around M38 and the strain of *B cereus*.

Discussion

These experiments have confirmed that supernatants prepared from *B pumilus* have toxic properties. Whether all the effects were caused by one substance has not been determined. The filtrable toxins seemed to be produced late in the bacterial growth cycle or possibly were released following cell injury or death. Brophy and Knoop² found that filtrates prepared from brain-heart infusion cultures of six *B pumilus* isolates (from guinea pigs with clindamycin induced enterocolitis) produced aggregation of Y1 adrenal cells within four hours and cell lysis after 18 hours. Our results cannot be compared directly with those of Brophy and Knoop, however, as the methods used for detection were not the same.

In our experiments the effects of the *B pumilus* supernatants were different from those previously described for *B cereus*.¹⁰ The variations in the effects of the supernatants of *B pumilus* were unexpected; in the original screening M38 had so little effect on the cell sheets after 24 hours it was chosen as the non-toxicogenic strain for further experiments; these showed that M38 supernatant was slow to act but lethal after incubation at 37°C for 96 hours. The cells treated with the *B pumilus* M11 supernatant showed a pronounced cytotoxic response before 48 hours but were still alive at 96 hours.

The reasons for the choice of Vero cells have already been given, but it is interesting to note that no cytopathic effect occurred with supernatants of *Ent agglomerans* or *B subtilis*. Thompson *et al* only examined the disruptive effect of *B cereus* in Vero cells for 12 hours and recorded cytotoxicity as the reciprocal of that dilution which resulted in complete loss of cytopathic activity.⁸

In 1930 Krontowski produced evidence of cellular regeneration following removal of

bacterial supernatant.¹² Working with *Corynebacterium diphtheriae* toxin and chick embryo cultures, he showed reduction in the area of tissue growth when diluted toxin was added to the growth medium; reversion to normal growth medium induced regeneration (equivalent in size to that of the control culture) within three subsequent passages. He also described the development of projections from some of the cells treated with diphtheria toxin. In our experiments regeneration occurred when the cytopathic *B pumilus* supernatant was replaced in the wells by growth medium, but did not occur when it was replaced by maintenance medium. If regeneration occurred at all, however, it was more likely to happen in response to the substitution with growth medium as the higher concentration of fetal calf serum in this medium is intended to support active growth rather than mere survival of the tissue culture.

These experiments do not provide direct evidence that the *Bacillus* species have a role in the aetiology of byssinosis, but the detection of these toxic effects justify further work. Furthermore, serum antibodies to these strains have been shown in cotton mill workers.¹³ One hypothesis is that toxin is produced at body temperature after inhalation of the bacteria. This would account for symptoms increasing during the shift¹⁴; the disappearance of symptoms when away from the mill might be explained by the regeneration of damaged tissue,¹⁵ if the inhaled toxin-producing bacteria had been overcome by host defences. This hypothesis does not explain the Monday onset (first working day) but there might be a quantitative factor or some host factor.

If this hypothesis is correct it would be interesting to investigate the role of iron in toxin production because the concentration is critical for the production of *C diphtheriae* toxin.¹⁶ Strippers and grinders who are responsible for the maintenance of the carding machines,¹⁵ and who might therefore have

greater exposure to iron particles and to damaged bacteria, are those most commonly affected by byssinosis. Obviously there is still much work to be done to test these hypotheses.

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