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REVIEW ARTICLE

Pantoea agglomerans: a mysterious bacterium of evil and good. Part I. Deleterious effects: Dust-borne endotoxins and allergens – focus on cotton dust

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Abstract

The ubiquitous Gram-negative bacterium *Pantoea agglomerans* (synonyms: *Enterobacter agglomerans*, *Erwinia herbicola*) is known both as an epiphytic microbe developing on the surface of plants, and as an endophytic organism living inside the plants. The bacterium also occurs abundantly in plant and animal products, in the body of arthropods and other animals, in water, soil, dust and air, and occasionally in humans. From the human viewpoint, the role of this organism is ambiguous – both deleterious and beneficial: on one side it causes disorders in people exposed to inhalation of organic dusts and diseases of crops, and on the other it produces substances effective in the treatment of cancer and other diseases of humans and animals, suppresses the development of various plant pathogens, promotes plant growth, and appears as a potentially efficient biofertilizer and bioremediator.

P. agglomerans has been identified as a predominant bacterium on cotton plants grown worldwide, usually as an epiphyte, rarely as pathogen. It is particularly numerous on cotton bract after senescence. During the processing of cotton in mills, bacteria and their products are released with cotton dust into the air and are inhaled by workers, causing respiratory and general disorders, usually defined as byssinosis. The most adverse substance is endotoxin, a heteropolymer macromolecule present in the outermost part of the cell wall, consisting of lipopolysaccharide (LPS) as a major constituent, phospholipids and protein. Numerous experiments carried out in last quarter of 20th century on laboratory animals and human volunteers supported convincing evidence that the inhaled endotoxin produced by *P. agglomerans* causes numerous pathologic effects similar to those elicited by cotton dust, such as the influx of free lung cells into airways and activation of alveolar macrophages which secrete mediators (prostaglandins, platelet-activating factor, interleukin-1, tumour necrosis factor) that cause the accumulation of platelets in pulmonary capillaries, initiating acute and chronic inflammation, resulting in endothelial cell damage and extravasation of cells and fluids into the lung interstitium. These changes cause bronchoconstriction, the decrement of lung function expressed as reduction of forced expiratory volume in one second (FEV_1) and/or diffusion capacity, increase in the airway hyper-reactivity and subjective symptoms such as fever, airway irritation and chest tightness. The conclusions from these experiments, performed mostly 20–30 years ago, did not loose their actuality until recently. This was because to-date no other cotton dust component had been identified as a more important work-related hazard than bacterial endotoxin. Although other microbial and plant constituents are also considered as potential causative agents of byssinosis, the endotoxin produced by *Pantoea agglomerans* and other Gram-negative bacteria present in cotton dust, is still regarded as a major cause of this mysterious disease.

Key words

Pantoea agglomerans, endotoxins, allergens, cotton dust, byssinosis, work-related symptoms, animal experiments

Abbreviations

CA-S – cell-derived mix of protein antigens and endotoxin, obtained by the extraction of bacterial mass with saline (0.9% NaCl); CCP – bacterial cells coupled with cellulose powder; CE-A – cell-bound endotoxin, obtained by the precipitation of bacterial mass with acetone (acetone powder); FEV_1 – forced expiratory volume in one second; KCS – killed cells suspension; LCS – live cells suspension; LPS-PW – lipopolysaccharide extracted by the phenol/water method; LPS-TCA – lipopolysaccharide obtained by extraction with trichloroacetic acid and precipitation with acetone; PAM – pulmonary alveolar macrophages. PMN – polymorphonuclear leukocytes; VECN – vesicular endotoxin-containing nanoparticles

A ubiquitous organism with many names and unusual properties. The species *Pantoea agglomerans* (Beijerinck 1888; Gavini et al. 1989) was created by combining three former species (*Enterobacter agglomerans*, *Erwinia herbicola*, and *Erwinia milletiae*) in a new one [1, 2]. It belongs to the family Enterobacteriaceae, which is included into Gammaproteobacteria class that comprises facultatively anaerobic and fermentative Gram-negative bacteria [3]. The species comprises non-capsulated, non-sporeforming straight rods measuring 0.5–1.0×1.0–3.0 μm, motile with peritrichous flagella (Fig. 1), usually yellow-pigmented [1].

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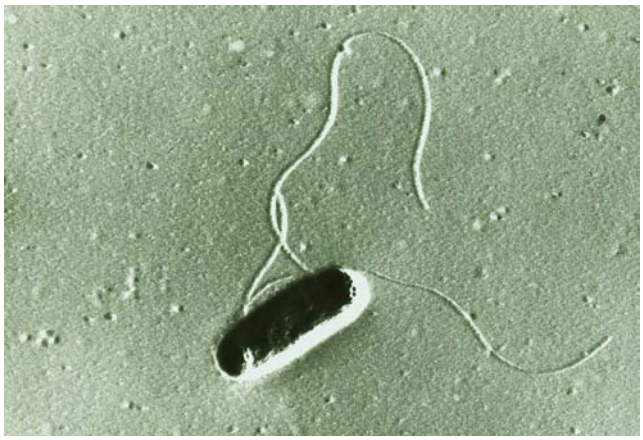

Figure 1. Electron micrograph of *Pantoea agglomerans*, preparation shadowed with silica oxide, × 40,000.
Source: Dr Barbara Urbanowicz, Laboratory of Electron Microscopy, Institute of Paediatrics, Collegium Medicum of the Jagiellonian University in Kraków, Poland

The generic name '*Pantoea*' is derived from the Greek word '*pantoios*', which means 'of all sorts and sources', and well reflects the ubiquitous occurrence of the bacterium in nature [1]. The specific name '*agglomerans*' meaning 'forming into a ball' derives from the unique property of the organism to form peculiar, elongated aggregates surrounded by a translucent exopolysaccharide sheath, called 'symplasmata', which play an important role in the colonization of the host plant by *P. agglomerans* (Fig. 2) [4, 5]. Earlier, the bacterium was classified under many names, such as: *Agrobacterium gypsophilae*, *Bacterium herbicola*, *Bacterium typhi flavum*, *Erwinia lathyri*, *Flavobacterium rhenanum*, *Xanthomonas trifolii* [6]. An excellent review article on *Pantoea* genus providing a comprehensive information on *P. agglomerans* has been published recently by Walterson and Stavrinides [7].

Pantoea agglomerans is probably one of the most widespread organisms in the world, at least in the sphere inhabited by humans. Originally a plant bacterium, it is known both as an epiphytic microbe developing on the surface of plants and as an endophytic organism living inside the plants [7, 8]. The bacterium also occurs abundantly in plant and animal products, in the body of arthropods and other animals, in water, soil, dust and air, and occasionally in humans [7, 9, 10]. From the human viewpoint, the role of this organism is ambiguous – both deleterious and beneficial: on one side, it causes disorders in people exposed to inhalation of organic dusts [11] and diseases of crops [12], and on the other, it produces substances effective in the treatment of cancer and other diseases of humans and animals [13], suppresses the development of various plant pathogens by antibiotic production and/or competition [7, 14], promotes plant growth by nitrogen fixation and other mechanisms [5, 7, 8], and appears as a potentially efficient bio-fertilizer and bio-remediator [7, 15]. Unraveling these problems is a fascinating experience for any scientist. To stimulate the interdisciplinary discussion on this mysterious bacterium we decided to prepare a series of 4 review articles, of which the first 3 present the deleterious effects of *P. agglomerans* (Part I: Endotoxins and allergens in cotton dust; Part II: Endotoxins and allergens in grain dust, other agricultural dusts and wood dust; Part III: Infections of humans, animals, and plants), whereas the last article (Part IV) presents the beneficial effects of this bacterium and concluding remarks.

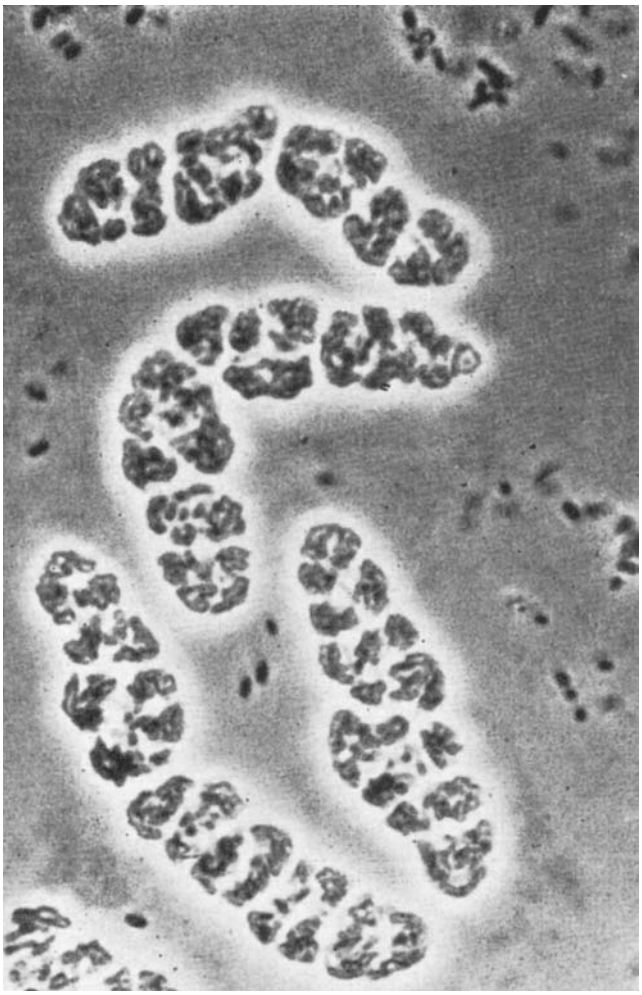

Figure 2. Phase-contrast micrograph of symplasmata with the characteristic 'sausage-shaped' aggregates formed by *P. agglomerans*, surrounded with an exopolysaccharide sheath, × 800. According to Bottone and Schneierson [4], with permission

Dust-borne endotoxins and allergens of *Pantoea agglomerans* – historical. In 1555, the Swedish bishop Olaus Magnus [16] described for the first time the deleterious effects of the fine dust released during grain threshing on the 'vital organs' of threshers. In 1713, the Italian physician Bernardo Ramazzini, known as 'the father of occupational medicine', in his book *De Morbis Artificum Diatriba* [17] gave a very accurate description of the symptoms occurring in grain handling workers. Although which specific agents caused the symptoms observed by both authors are not known, it cannot be excluded that they were elicited by endotoxin of *Pantoea agglomerans*, the major disease factor present in fresh (not only in mouldy or overheated) grain. The first discovery documenting the important role of Gram-negative bacteria in evoking the disorders caused by the inhalation of organic dusts was made by US scientists in the USA in 1942 [18, 19] when the world was embroiled in the most calamitous war ever experienced. At that time, Neal et al. [18] noticed the presence of respiratory and general symptoms in mattress makers working with a low grade, stained cotton. In the subsequent article by Schneiter et al. [19], the authors reported the mass occurrence of such symptoms in the workers exposed to cotton dust from 25 US states and 2 Canadian provinces. The symptoms included cough, chest

tightness, chills, fatigue, fever, and other symptoms ascribed today to the acute stage of byssinosis (a disease caused by exposure to cotton dust), or to organic dust toxic syndrome (ODTS, toxic pneumonitis). The authors identified the specific causative agent of the disease as a fermentative Gram-negative bacterium which they had isolated from cotton and cotton dust in the concentrations of 4.0×10^3 – 5.7×10^9/g, and described it as 'cotton bacterium' or 'mucoid bacterium'. They tentatively classified this microorganism as '*Aerobacter*' (an old synonym of *Enterobacter*), demonstrated its pathogenic properties by experiments on animals and humans, and correctly proposed the main role of an 'endotoxin-like substance' produced by this bacterium in evoking the disease [19]. The 'cotton bacterium' was later identified as '*Aerobacter cloacae*' [20], although it was most probably *P. agglomerans*, as the identification methods available in 1942 did not allow for proper identification of isolated strains. This problem may be solved if the strains from 1942 persisted until now, as they could be easily identified by genetic methods, and some inconsistencies between metabolic properties of these strains and standard *P. agglomerans* strains, such as gas production from carbohydrates, could be clarified. World War II prevented further development of the 'endotoxin hypothesis' which was then forgotten for many years. About 20 years later the hypothesis was revived by the Italian scientists Pernis et al. [21] and Cavagna et al. [22] who demonstrated by well-designed experiments the role of bacterial endotoxin in causing diseases due to exposure to cotton dust; however, without attributing this hypothesis to any species of Gram-negative bacteria. Cavagna [23], using biological tests in the skin of rabbits, quantified for the first time the 'endotoxin-like substances' in settled and airborne cotton dust. He obtained reliable figures which showed an excellent correlation with the frequency of symptoms in exposed workers, and essentially presented a good agreement with the later introduced sensitive *Limulus* test.

Fifteen years later, in the second half of 1970s, the real development began of studies on the significance of the main role of bacterial endotoxin in causing byssinosis and other diseases caused by exposure to cotton dust with *P. agglomerans,* due to research by the prominent scientists: Ragnar Rylander from Sweden, the late Janet J. Fischer (1923–2007), the late Robert Burrell (1933–2012), Robert R. Jacobs, Anthony J. DeLucca, R. Clark Lantz, Meryl H. Karol, and Stephen A. Olenchock, all from the USA, as well as many others whose contributions were certainly not less than those mentioned above. The studies conducted by Rylander and other scientists demonstrated an abundant occurrence of Gram-negative bacteria in cotton dust with *P. agglomerans* as a major component, an abundant presence of bacterial endotoxin in cotton and cotton dust (having been detected by a sensitive *Limulus* test, based on the clotting of the blood of the primitive marine arthropod *Limulus polyphemus* in the presence of trace amounts of endotoxin), and the potent effects of *P. agglomerans* endotoxin or whole cells on the respiratory system of experimental animals and human volunteers [24, 25, 26, 27, 28, 29]. These findings, often new to science, were published in scientific periodicals and presented at special scientific meetings devoted to the problems of the health effects of organic dusts, and organized in the last quarter of 20th century, such as: the Cotton Dust meetings organized yearly as a part of the Beltwide Cotton Production Research Conferences in the southern states of the USA, the

Figure 3. The 3rd Skokloster International Workshop 'Causative Agents for Organic Dust Related Disease', Skokloster, Sweden, 6–9 April 1992. Professor Ragnar Rylander (in the front, in white coat), chief organizer of the meeting, discusses with participants the problems of the organic dusts effects on the shore of a scenic lake. Photograph by J. Dutkiewicz

Skokloster Conferences in a small locality at the scenic lake region of Stockholm-Uppsala in Sweden (Fig. 3), and the Endotoxin Inhalation Workshop in Clearwater, Florida, in the USA.

Probably never before nor never after has so much attention been paid to *P. agglomerans* as between 1975–2000. The endotoxin of this bacterium was identified, mainly in Sweden and the USA, as an important cause of disorders following exposure to cotton dust [25, 28, 29], and recommended for experimental study of the etiopathogenesis of these disorders [30]. At approximately the same time in Lublin, a medium-size city in eastern Poland, the endotoxin and allergens of *P. agglomerans* were implicated as the causative agents of disorders in persons and farm animals exposed to grain dust and other agricultural dusts [31, 32, 33, 34, 35], which will be presented in detail in the next article of the *Pantoea*-series.

***Pantoea agglomerans* endotoxin and its outstanding biological activities.** Bacterial endotoxin is a heteropolymer macromolecule present in the outermost part of the cell wall of Gram-negative bacteria, consisting of lipopolysaccharide (LPS) as the major constituent, phospholipids and protein. The term 'endotoxin' is often used interchangeably with 'LPS', but this is not entirely correct because 'endotoxin' is a wider term comprising LPS with associated proteins and phospholipids, which is more adequate in relation to particles present in dusts. The endotoxin molecule consists of 3 regions: lipid A, possessing most of the biological activities, core oligosaccharide and O-polysaccharide side chains, possessing antigenic properties [36]. Endotoxins reveal a unique spectrum of biological activities, usually common for all Gram-negative bacteria, but often showing species-related differences [36]. They are isolated mostly with 2 methods: by extraction in trichloroacetic acid (TCA), followed by precipitation with acetone according to Boivin et al. [37], and by phenol-water (P-W) extraction according to Westphal et al. [38]. The injection of endotoxin produced by *P. agglomerans* or other Gram-negative bacteria into experimental animals causes a wide spectrum of non-specific pathophysiological reactions, such as fever, changes in white blood cell counts, disseminated intravascular coagulation, hypotension, shock and death [39].

The first characteristics of the biological activity of endotoxins isolated from *P. agglomerans* was presented by Dutkiewicz in 1976 [31]. The TCA-extracted endotoxins (LPS-TCA) of 10 strains of *P. agglomerans* isolated from airborne grain dust and from human and animal sources were lethal for mice, and evoked primary inflammatory lesions and local Shwartzman reactions in the skin of rabbits (Fig. 4) in doses comparable to those of known enterobacterial endotoxins. The tested endotoxins of *P. agglomerans* also elicited haemorrhagic lesions in the skin of rabbits after mixing with adrenaline, according to Thomas [40], and were lethal for 10-day chick embryos. Dutkiewicz concluded that *P. agglomerans* produces a potent endotoxin which should be considered as a potential cause of work-related disorders among grain workers and other workers exposed to organic dusts [31]. In subsequent years, the endotoxic properties of *P. agglomerans* were confirmed by Rylander and Lundholm [24], Helander et al. [41, 42] and Salkinoja-Salonen et al. [43].

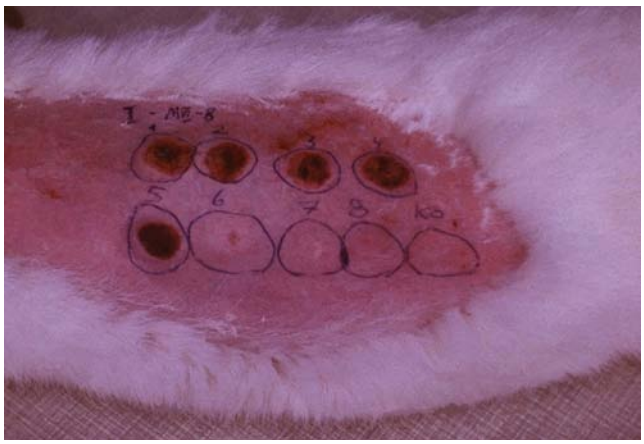

Figure 4. Local Shwartzman reaction in rabbit skin, photographed 48 hrs after intradermal injection with graded amounts (site 1=200 µg, site 8=1.56 µg) of the preparation tested for endotoxic properties (in this case, LPS-TCA from *P. agglomerans* strain M-6-8 isolated from the air of grain mill), and 24 hrs after intravenous challenge with a known endotoxin (in this case, *Salmonella typhi* O901 Difco endotoxin). Positive reactions appear as haemorrhages, highly visible up to site 5 injected with 12.5 µg of the preparation. According to Dutkiewicz [31]

These authors demonstrated that the inhalation exposure of guinea pigs to live cell suspensions (LCS) or endotoxins (LPS-PW) from *P. agglomerans* strains isolated from cotton, after 24 hours caused a significant influx of polymorphonuclear leukocytes (PMNs) and pulmonary alveolar macrophages (PAMs) into the lungs. The LPS-PW preparations of *P. agglomerans* were also lethal for mice and pyrogenic for rabbits [42]. The inhalation exposure of guinea pigs to the lipid A fraction obtained from LPS-PW of *P. agglomerans* caused an influx of PMNs and PAMs into the lungs, and an increase in body temperature, whereas the polysaccharide fraction of LPS-PW had no effect [44].

The endotoxic potency of the LPS preparations from *P. agglomerans* proved to be essentially similar to that shown by LPSs produced by the majority of Enterobacteriaceae species (e.g. *Citrobacter freundii*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *Salmonella typhi*, *Salmonella typhimurium*), or the Pseudomonadaceae species (e.g. *Pseudomonas putida*) [31, 41, 42, 43], but greater compared to LPSs from some other non-enterobacterial Gram-negative bacteria, such as *Agrobacterium* sp. and *Xanthomonas sinensis* [41, 42] or *Acinetobacter calcoaceticus* and *Alcaligenes faecalis* [45]. Moreover, in some experiments, LPSs from *P. agglomerans* showed a greater biological activity compared to those obtained from strains of *Escherichia coli*, used in many laboratories as a standard endotoxin-producing bacterium. Thus, Lewis [46] demonstrated that *P. agglomerans* LPS appeared to be 170 times more potent in inducing an inflammatory monokine, interleukin 1 production by mouse alveolar macrophages *in vitro* than *Escherichia coli* LPS. In an earlier study, Helander et al. [41] noted a distinctly greater migration of PMNs and PAMs into the lungs of guinea pigs exposed by inhalation to *P. agglomerans* LPS, compared to those exposed to *E. coli* LPS. In another study, Gatty et al. [47] found that the respiratory response of guinea pigs to LPS from *E. coli* was less than those obtained to LPSs from *P. agglomerans* and *Pseudomonas syringae* isolated from cotton dust, which were almost identical.

Rylander demonstrated in 1988 [48] that the cell-bound endotoxin of *P. agglomerans* (CE-A), resembling that naturally occurring in dust, showed stronger pulmonary toxicity than the isolated and purified lipopolysaccharides obtained by P-W extraction. This could be explained by the presence of particles enhancing, by their large size and shape, macrophage activation [11, 49], as well as by the adjuvant action of proteins and other cell wall constituents which do not occur in the purified lipopolysaccharide preparations. In the Workgroup Reports from the Endotoxin Inhalation Workshop organized between 28–30 September 1987 in Clearwater, Florida, USA [50], native forms of cell-bound endotoxin 50:50, partly disrupted by sonication and containing 50% of fragments and 50% of whole cells (as assessed by electron microscopy), were proposed for experiments. Accordingly, in the summary of this Workshop, Rylander and Burrell [11] recommended using the cell-bound endotoxin of *P. agglomerans* as a reference standard preparation for the experimental assessment of the effects of occupational exposure to dust-borne endotoxin, instead of the standard preparations of isolated lipopolysaccharides, mostly of *Escherichia coli* used in the pharmaceutical industry.

This attitude was later confirmed by the works of our Polish-American group: firstly, by the discovery that endotoxin produced by *P. agglomerans* and other Gram-negative bacteria occurs in organic dusts from grain, wood and other materials in the form of the vesicular nanoparticles (Fig. 5) [51, 52], secondly by demonstrating that the isolated vesicular endotoxin-containing nanoparticles (VECN) exert stronger biological effects than the purified lipopolysaccharides [53], and thirdly by proving that the saline extracts of the cell mass (CA-S) of *P. agglomerans*, resembling a native mix of protein antigens and endotoxin, show potent biological effects, much stronger compared to isolated lipopolysaccharides of *P. agglomerans* or to other adverse microbial agents occurring in organic dusts [54, 55, 56, 57]. These works will be discussed in detail in the next article of the *Pantoea*-series.

An important role in the mediation of reactions elicited by endotoxin is played by the toll-like receptors (TLR), mostly TLR4, as well as CD14 (cluster of differentiation 14) and MD2 (myeloid differentiation factor 2) receptors. The inhaled endotoxin interacts with the CD14/TLR4/MD2 receptor complex in many cell types, but especially in monocytes, dendritic cells, macrophages and B cells, which promotes the secretion of pro-inflammatory cytokines, nitric oxide, and eicosanoids (oxygenated derivatives of 3 different

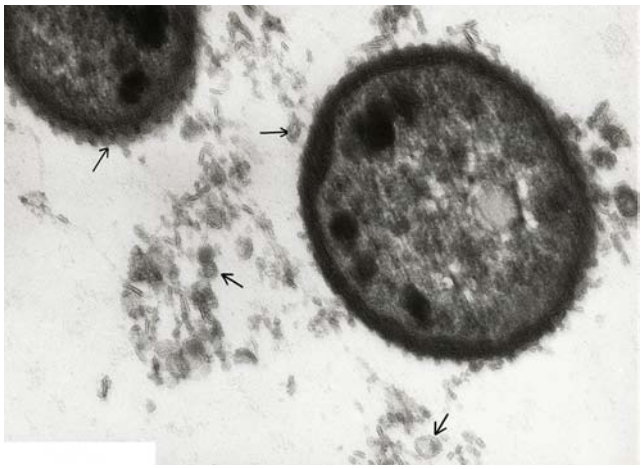

Figure 5. Electron micrograph of two bacterial cells corresponding to *Pantoea agglomerans* in the lumen of a wood cell of American basswood (*Tilia americana*), × 100,000. Peeling of the vesicular endotoxin-containing nanoparticles (VECN) in the upper left cell, and the detached VECN showing increased volume are marked with arrows. According to Dutkiewicz et al. [52]

20-carbon fatty acids, including leukotrienes, eoxins, prostaglandins, prostacyclins, and thromboxanes). As a result, inflammation develops at the site of action as well as in other parts of the body [58, 59]. The recent study by Golec et al. [60] demonstrated a significant increase of TLR2, TLR4, and TLR8 in the lung tissue of mice after inhalation exposure to saline extract (CA-S) of the *P. agglomerans* cells. The genes regulating TLR4, as well as CD14 and MD2 receptors, are important in the inflammatory response to endotoxin [58].

Inflammation caused by endotoxin results in a decrease in the reactivity to atopic allergens by promoting a TH1-type immune response, interfering with TH2-type immune response associated with atopy. As a result, the risk for atopic, IgE-mediated sensitization is less among children raised on farms and exposed to high levels of endotoxin, as demonstrated in Germany and Switzerland [61]. In a study carried out on a group of 1,614 Norwegian farmers, Eduard et al. [62] showed that exposure to endotoxin and fungal spores has a protective effect on atopic asthma, but may induce non-atopic asthma in farmers.

***Pantoea agglomerans* as a predominant bacterium in cotton dust.** *Pantoea agglomerans* has been identified as a cause of opportunistic bacterial seed and boll rot of cotton (*Gossypium hirsutum*) grown in the field [12], but it usually occurs on cotton as an epiphyte. As such, it was identified as a predominant bacterium on cotton plants grown worldwide. According to DeLucca and Palmgren [63], reporting results from the USA, this species prevailed on cotton bracts (Fig. 6) where the bacterial population was highest, forming on average 26.2% of the total bacterial counts, ranging from 3.0 – 1,000.0×10^6/g. *P. agglomerans* also prevailed on cotton leaves, forming 22.9% of the bacterial counts, ranging from 0.09 – 7.6×10^6/g, and was the second most numerous on fibre (after opening of the locks) forming 21.7% of the bacterial counts, ranging from 0.3 – 9.0×10^6/g.

The concentrations of Gram-negative bacteria and endotoxin are especially high on cotton from warm, humid regions [64]. The levels of Gram-negative bacteria, including *P. agglomerans*, and endotoxin on bract increase from 10 – 100-fold after the first killing frost and senescence, and may increase also after rain [49, 63, 64, 65]. High levels of

Figure 6. Cotton boll containing seeds and fibre, surrounded by bracts (BR). Cotton bracts contain abundant concentrations of *Pantoea agglomerans*, mostly after senescence

P. agglomerans and endotoxin produced by this bacterium which are present on senescent bract, may release into the air during carding causing a risk of the inhalation exposure to cotton workers [63]. Thus, the monitoring of climatic variables is important for determining the best time to harvest cotton, just before the dramatic increase in *P. agglomerans* and endotoxin concentrations [64].

The predominance of *P. agglomerans* on cotton plant and in cotton dust has also been confirmed by other authors. Berni et al. [66] found that this species was predominant on cotton bract after senescence and was the second most common on fibre. Millner et al. [67] found that *P. agglomerans* was a predominant bacterium on cotton harvested in Texas, USA, where it occurred abundantly in the concentrations up to 10^7 cfu/g. Fischer and Foarde [68] identified *P. agglomerans* as a dominant constituent of the total Gram-negative population of cotton from various parts of world (Syria, Mali, Chad, Tanzania, USA), usually forming 50–80% of the total, while the second most numerous species *Ps. syringae* formed about 25% of the total.

The dominancy of *P. agglomerans* among bacteria found on cotton results in the abundant occurrence of this bacterium in the airborne dust present in cotton mills. This was well documented by Haglind et al. [25] who found that the concentration of Gram-negative bacteria in bale and carded cotton from Peru, Turkey, Russia, and the USA, processed in 5 Swedish mills was 10^2–10^8 cfu/g with *P. agglomerans* forming 17 – 99% of the total, in most cases over 75%. In these mills, the concentration of airborne Gram-negative bacteria was within the range 0.6 – 112.0 cfu×10^3/m^3, being strikingly lower in the spinning areas than in the carding rooms. *P. agglomerans* was the absolute dominant, forming between

47 – 93% of the viable airborne Gram-negative bacteria. Similarly, Lacey and Lacey [69] reported that *P. agglomerans* was one of predominant bacteria in the air of English cotton mills. Also, Doctor et al. [70] found an abundant occurrence of *P. agglomerans* in the air of gin house in India, where it was the dominant species among Gram-negative bacteria. This was associated with high concentrations of endotoxin ranging between 1.52 – 2.77 μg/m^3.

A convincing demonstration of the role of *Pantoea agglomerans* and other endotoxin-producing Gram-negative bacteria in the etiology of occupational disorders due to exposure to cotton dust. The most important occupational disease due to exposure to cotton dust, and also to dust from flax and hemp, is byssinosis, characterized in the acute stage by chest tightness and fever usually appearing on Mondays, after the weekend break, and in the chronic stage by respiratory impairment. Although the symptoms of byssinosis were described several centuries ago [71], none of the individual components of the cotton dust were identified as the specific cause of this disease until the 1970s. Between 1975–1985, firm experimental and epidemiological proof was provided of the significant role of exposure to Gram-negative bacteria and the endotoxin they produced in causing pulmonary inflammatory reactions, and symptoms of occupational disease caused by the inhalation of cotton dust. Rylander and Snella [72] found that Gram-negative bacteria prevailed among total bacteria isolated from various cotton dusts. The authors demonstrated a significant correlation between the concentration of Gram-negative bacteria and endotoxin in different cotton bracts and dusts, and the increase in the number of polymorphonuclear leukocytes and macrophages in the lungs of guinea pigs exposed to the inhalation of aerosol from water extracts of these dusts. Rylander and Lundholm [24] demonstrated that the examined samples of cotton plants, bale cotton and cotton dust were contaminated primarily by Gram-negative bacteria, mainly of *Pantoea*, *Enterobacter*, *Pseudomonas*, and *Agrobacterium* genera. Similarly, as shown in the paper cited above in relation to cotton dust extracts, the inhalation exposure of guinea pigs to cell suspensions of *P. agglomerans*, *E. cloacae*, *K. oxytoca* and *Ps. syringae* isolated from cotton, caused a significant influx of polymorphonuclear leukocytes and macrophages into the lungs 24 hours post-exposure, and in similar quantities for all the species tested. No effects were recorded after exposure to *Agrobacterium* and *Bacillus* cells.

Rylander et al. [73] demonstrated a significant correlation between the concentration of Gram-negative bacteria in bale cotton processed in 23 mills in the USA and the decrement of lung function in the exposed workers expressed as FEV_1. This result corresponds well with those obtained by Cinkotai et al. [74] and Haglind et al. [25], who found a significant correlation between the number of airborne Gram-negative bacteria, consisting mainly of *P. agglomerans* [25], and the prevalence of byssinosis symptoms in epidemiological studies carried out in different cotton mills. Rylander [75] stressed the similarity between the different symptoms related to cotton and flax mill exposure, such as mill fever, histamine release, airway broncho-constriction, and chronic bronchitis to those induced by the inhalation of Gram-negative bacteria and LPS, and suggested the important role of latter agents in the etiology of byssinosis. Since then, a marked interest grew in studies on *P. agglomerans* and other endotoxin-producing Gram-negative bacteria as purported agents of occupational disorders related to cotton dust.

In 1987, Rylander [49] maintained his view on the important role of endotoxins in causing byssinosis and other disorders related to exposure to cotton dust, and stressed a need for the monitoring of concentrations of airborne endotoxins in the cotton industry for better protection of the workers. According to him, the primary target for inhaled endotoxin is the alveolar macrophage which secretes mediators (prostaglandins, platelet-activating factor (PAF), interleukin-1, tumour necrosis factor (TNF)) causing an accumulation of platelets in the pulmonary capillaries, initiating an acute and chronic inflammation. This view is in accordance with the results obtained by Milanowski [54, 55] who found in experiments carried out *in vitro* and *in vivo* potent effects of the *P. agglomerans* endotoxin preparations (LPS-TCA, CA-S) on the morphology and function of guinea pig alveolar macrophages (Figs. 7–8).

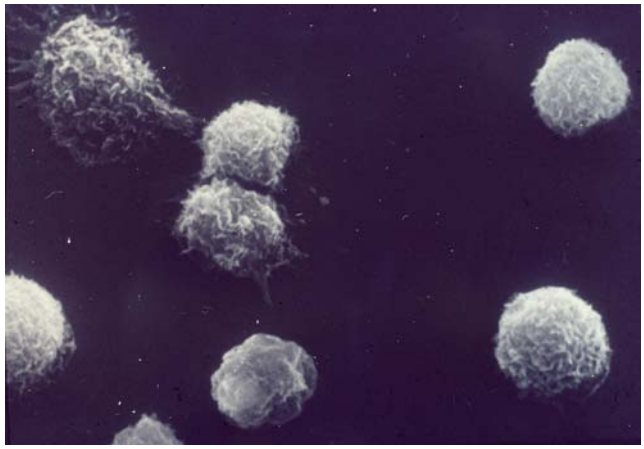

Figure 7. Scanning electron micrograph of alveolar macrophages from a normal guinea pig, not exposed to endotoxin, × 2,000. Note rounded shape and smooth surface with small pseudopodia. According to Milanowski [55]

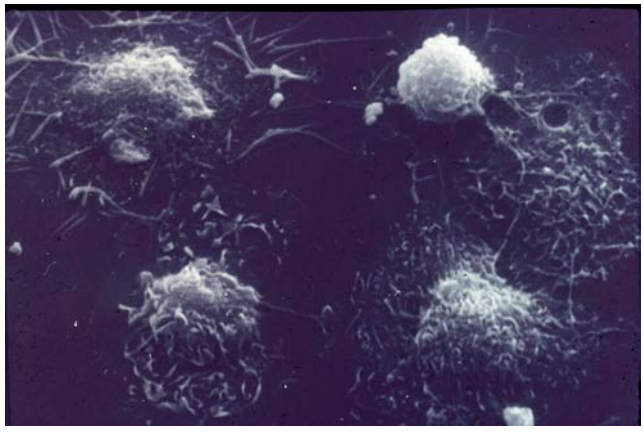

Figure 8. Scanning electron micrograph of alveolar macrophages from a guinea pig exposed to *Pantoea agglomerans* endotoxin-containing preparation (CA-S), × 2,000. Note rough surfaces, blebs, filopodia and rufflings in their surfaces. According to Milanowski [55]

Twenty years later, Rylander [58, 76] expressed an opinion that endotoxin has been firmly established as a major causative agent for the inflammatory disease caused by the occupational exposure to organic dusts, causing inflammation manifested by cough, airway irritation, chest tightness, decrease in pulmonary function, and, at higher exposure levels – toxic pneumonitis (ODTS). Repeated

exposure to endotoxin may induce a tolerance or adaptation manifested by the disappearance of symptoms in exposed workers [76].

In conclusion, the view about the major role of endotoxin, produced mostly by *P. agglomerans*, in causing byssinosis remains actual to this day, and determines the main directions of prevention, aimed at the reduction of dust and endotoxin in the air of cotton mills, mostly cardrooms.

Animal experiments proving the potential role of *P. agglomerans* endotoxin in causing cotton dust-related disorders. From 1980–1995, scientists from different centres, mostly located in the USA, studied the effects of the exposure to various preparations obtained from *P. agglomerans* strains isolated from cotton dust (LPS obtained by P-W extraction, cell-bound endotoxin obtained by precipitation with acetone, whole cells) or, for comparison, to cotton dust itself, in different animal or cell culture models, using doses mimicking real exposure of cotton mill workers. Important results were obtained by scientists from University of Pittsburgh where Karol et al. [77] demonstrated a similar respiratory response in guinea pigs exposed to inhalation of cotton dust and CE-A of *P. agglomerans*, manifested by a pattern of 'reflex restriction', comprising an increase in respiratory frequency and decrease in tidal volume and plethysmograph pressure. The sole difference concerns airflow obstruction which occurred only in animals exposed to cotton dust. In the next experiment, Karol et al. [27] compared 3 endotoxin-containing preparations of *P. agglomerans*: LPS, CE-A and CCP. All preparations elicited the 'rapid shallow breathing' in guinea pigs exposed by inhalation, resembling this after exposure to cotton dust. The strongest response in respiration frequency and airflow interruption was noted after the application of cells coupled with cellulose powder. Similarly, Fischer et al. [78] noted an increase in respiratory frequency, and decrease in tidal volume in guinea pigs subjected to the inhalation of *P. agglomerans* and *Ps. syringae* endotoxins. The responses of the animals to endotoxins and to cotton dust were very similar, indicating the etiological role of endotoxin in byssinosis. In the following experiment carried out 2 years later (1988), Spear et al. [79] found that artificial dust prepared by growing of *P. agglomerans* on a cellulose powder (CCP) elicited in the guinea pigs exposed to its inhalation the same pattern of response seen before with cotton dust having similar endotoxin content: increased respiratory frequency, decreased tidal volume, the occurrence of airflow interruption and histopathologic changes in the lung. Also in 1988, Olaniran and Karol [80] exposed guinea pigs for 12 months to a large concentration of airborne cotton dust (21 mg/m^3) and noted haematologic changes and the formation of specific antibodies, not only to cotton dust itself, but also to *P. agglomerans*, the most prevalent Gram-negative bacterium in the dust. For the prevention of disorders caused by the inhalation of cotton dust, the results obtained by Griffiths-Johnson et al. [81] are important. They demonstrated that treatment of the *P. agglomerans* CE-A (preparation 50:50) with NaOH in ethanol removed pulmonary toxicity, when compared with an untreated cell preparation. This finding supports earlier studies in which base treatment decreased the pulmonary toxicity of cotton dust.

In 1994, Ryan et al. [29] from the Pittsburgh Center demonstrated the similarity of pulmonary reactions in 2 lines of mice exposed to the inhalation of cotton dust and LPS-PW from *P. agglomerans* in doses corresponding to real exposure in cardrooms (45 mg/m^3 and 2.4 μg/m^3, respectively). In the endotoxin-sensitive C3HeB/FeJ mice, the exposure both to cotton dust and *P. agglomerans* LPS caused neutrophil influx and TNF release, whereas in endotoxin-resistant C3H/HeJ mice no inflammation or TNF release resulted from the inhalation of LPS, and only minimal inflammation and TNF release were noted following exposure to cotton dust. These results suggest a major role for endotoxin in acute inflammation and TNF release induced by cotton dust inhalation, with a minor role for other components in cotton dust.

Scientists from the University of West Virginia in Morgantown subjected Syrian golden hamsters to inhalation of *P. agglomerans* LPS at the concentration of 4 μg/m^3, corresponding to real exposure of cotton workers, and examined thereafter by electron microscopy morphometric changes in the lungs of exposed animals and release of various inflammatory mediators. According to the authors, the primary site of inhaled *P. agglomerans* endotoxin appeared to be the endothelial cells. Significant ultrastructural alterations in the endothelium of distal lung were found in exposed animals in the form of focal disruption, intercellular oedema, subendothelial and intraendothelial vacuolization, cytoplasmic blebbing, and increases in the numerical density of pinocytotic vesicles, both in capillary endothelium and Type I alveolar epithelial cell cytoplasm (Fig. 9) [26]. The authors also recorded other changes in the distal lung of the exposed animals: a significant increase in the numbers and volume density of polymorphonuclear neutrophils (PMN) and platelets, a decrease in volume density of red blood cells in septal capillaries, increase in the volume of cellular interstitium of distal capillary septa and in the volume of type I epithelial cells, increase of pulmonary capillary permeability, and a significant decrease in total lung volume [26, 82, 83]. The statistically significant increases in serum

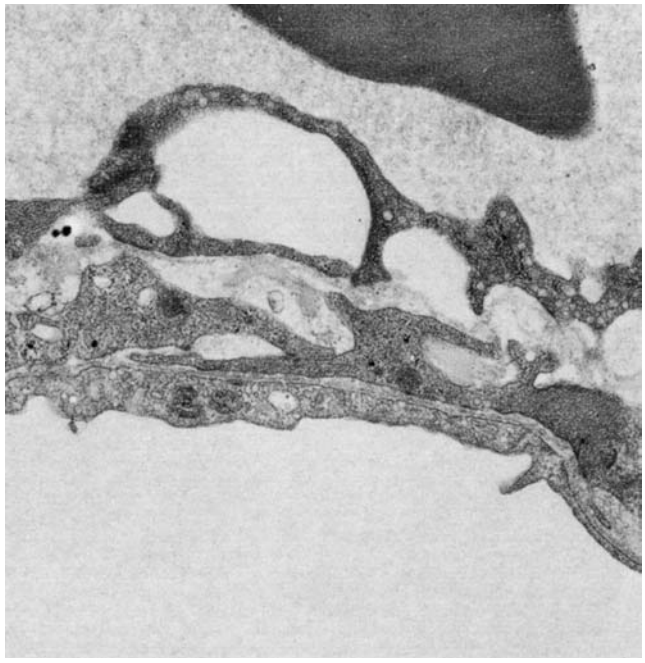

Figure 9. Electron micrograph of distal lung of a hamster exposed to inhalation of LPS-PW from *P. agglomerans,* showing foci of vacuolar subendothelial intercellular oedema, × 18,000. According to Lantz et al. [26], with permission

concentrations of prostaglandins: prostaglandin E_2 (PGE_2) and thromboxane B_2, but not prostaglandin PGF_2, were noted in exposed animals. Inactivation of complement or prostaglandins production abolished the decrease in lung volume and increased capillary PMN and platelets, whereas inactivation of complement, but not prostaglandins, blocked any increase in numerical density of endothelial pinocytotic vesicles. Any inactivation had no effect on the increase in cellular interstitium volume. According to the authors, the results showed that complement and/or prostaglandins mediate many, but not all, effects of *P. agglomerans* endotoxin on the lungs, and that the signal from inhaled endotoxin is probably transmitted across the air-blood barrier by vesicular transport across the epithelium [82]. Lantz et al. [83, 84] demonstrated the very important role of PAF in morphometric and physiologic responses of hamsters to aerosols of *P. agglomerans* LPS. Pretreatment of hamsters with PAF inhibitor decreased the PAF-mediated effects of exposure to endotoxin, such as damage to pulmonary capillary endothelial cells, increases in capillary permeability, and infiltration of neutrophils into airways. This experiment confirmed the results of an earlier collaborative study by Rylander et al. [85] on the large role of PAF produced by alveolar macrophages as the mediator of many, but not all, changes induced by inhaled endotoxin. Keller et al. [86] demonstrated that the inhalation exposure of hamsters to lipid A from *P. agglomerans* LPS caused measurable pulmonary oedema. According to the authors, this was induced by the activation of alveolar macrophages which metabolized lipid A, and initiated the physiological events which brought about the oedema.

Other experimental research work carried out by scientists provided further support for the major role of *P. agglomerans* endotoxin in causing detrimental response to cotton dust. Willoughby et al. [87], from the Johns Hopkins University School of Medicine in Baltimore, USA, demonstrated that the *P. agglomerans* LPS inhaled by rabbits induced pulmonary inflammation and fever, in association with release of IL-1, fibroblast growth factor and cytotoxic factors from alveolar cells. According to the authors, the latter factors may participate in the combined destruction of pulmonary parenchymal tissue. Fogelmark et al. [88] from the University of Gothenburg in Sweden, demonstrated, as a complementation of earlier works in this center, that the inhalation exposure of guinea pigs to CE-A of *P. agglomerans* caused a significant increase in pulmonary inflammatory cells (macrophages, lymphocytes, neutrophils, eosinophils) which appeared at first in lung walls after 4 hours, and than migrated into the airways, attaining a peak at 24 hours post-exposure, detectable by bronchoalveolar lavage (BAL). Elissalde and Beier [89] from College Station, Texas, USA – similar to Burrell et al. earlier [82] in hamsters – demonstrated that LPS of *P. agglomerans* stimulated isolated rat macrophages to produce and release arachidonic acid metabolites (eicosanoids), namely prostaglandins (PG): 6 keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, PGD_2, PGA_2, and PGB_2, and thromboxane B_2. They expressed an opinion that some of the acute pulmonary changes observed in humans following inspiration of cotton dust contaminated with *P. agglomerans* endotoxin could be caused by an increased release of these biologically active compounds.

DeLucca et al. [90] from the Southern Regional Research Center of USDA-ARS in New Orleans found that LPS from *P. agglomerans* bound to sheep pulmonary surfactant *in vitro,* and significantly altered its surface tension properties. According to the authors, such a reaction may change the physiological properties of surfactant *in vivo*, impair pulmonary function, and be a possible mechanism for the pathogenesis of byssinosis. In a later study, DeLucca and Brogden [91] demonstrated that the *P. agglomerans* LPS complexes with, and neutralizes, an ovine surfactant fraction responsible for enhancing the bactericidal capability of serum, thus weakening the animal immunity.

Cloutier and Rohrbach [92] from the University of Connecticut, and Cloutier and Thrall [93], reported that the effects of *P. agglomerans* endotoxin on the electrophysiologic and ion transport properties of the canine tracheal epithelium are lower compared to tannin or the cotton bract extract. However, in a later study, Cloutier et al. [94] demonstrated that *P. agglomerans* is capable of inducing the release of an unknown mediator from rabbit alveolar macrophages in sufficient amounts to alter the ion transport properties of the airway epithelium. Nevertheless, the results obtained by the latter authors suggest that the response of airways to cotton dust is probably a complex reaction, and that possibly some components of cotton dust other than endotoxin may contribute to the final adverse effect.

Experimental exposure of humans and human cells support the important role of endotoxin and *Pantoea agglomerans* in causing disorders related to cotton dust exposure. Haglind and Rylander [95] found in cotton workers and student volunteers exposed to the inhalation of cotton dust in an experimental cardroom, a significant correlation between the concentration of airborne endotoxin and the FEV_1 decrement during the working shift. This relationship was more pronounced in workers who smoked, which suggests that smoking predisposes to enhanced response to inhaled endotoxin. In exposed students without prior exposure to cotton dust, subjective symptoms were noted several hours after exposure, such as fever, chest tightness and cough. In subsequent research, carried out in workers from cotton mills exposed in an experimental cardroom to dust from cotton originating from different geographical locations in the USA, Rylander and Haglind [96] found again a significant correlation between the concentration of airborne endotoxin, but not dust, and the FEV_1 decrement, as well as an increase in blood neutrophils, but not platelets, during the working shift. A significant correlation was also found between the concentration of airborne endotoxin and the occurrence of subjective symptoms (chest tightness, breathing difficulties). According to the authors, the results suggest that the amount of airborne endotoxin determines the risk for the workers of developing the byssinosis syndrome, including mill fever, chest tightness, and acute decrements in FEV_1. The authors are right in assuming that the observed effects are not caused solely by pure LPS, but are probably due to a synergistic interaction with other agents, such as cell wall proteins from the Gram-negative bacteria, or other particulates in cotton dust. Castellan et al. [97] from the NIOSH, Morgantown, West Virginia, observed in volunteers experimentally exposed to cotton dust from a carding machine, a highly significant dose-response relationship ($P<0.0001$) between the concentration of airborne endotoxin and decrease in FEV_1, but not between the concentration of airborne dust and decrease in FEV_1. These results are in line with those

mentioned above, and strongly support the hypothesis that endotoxin has a causative role in the acute pulmonary response to inhaled cotton dust.

The endotoxin present in the cotton dusts mentioned above was not necessarily produced by *P. agglomerans;* however, the following studies do suggested such a relationship. Bake et al. [98] and Rylander et al. [28] found that healthy humans (student volunteers) subjected to the inhalation of LPS-PW and CE-A of *P. agglomerans* strain isolated from cotton at levels comparable to exposed cotton mill workers, showed a significant decrease in FEV_1 similar to that found in cardroom workers, as well as a significant decrease in carbon monoxide diffusion (DLCO), a significant increase in airway hyper-reactivity (measured by methacholine challenge 4 hours post-exposure), and subjective symptoms (fever, airway irritation and chest tightness). According to the authors [28], the reduction in DLCO may be due to inflammatory response (endothelial cell damage and extravasation of cells and fluids into the lung interstitium) after exposure to airborne endotoxin. The recorded adverse effects were much stronger after exposure to CE-A, compared to LPS-PW, most probably because CE-A was much more similar to environmental endotoxin present in the dust than purified LPS. The threshold doses causing symptoms, standardized by the *Limulus* test, were equal to 30 μg of LPS-PW or 300 μg of CE-A. The results of this research explained the negative results obtained earlier by Jamison and Lowry [99] from the University of Belfast in Northern Ireland, who did not observe a drop in spirometric values and occurrence of symptoms in human volunteers exposed to *P. agglomerans* LPS-PW in the doses of 5 μg and 12 μg, which were simply too low to evoke any reaction (except for a significant drop in DLCO after exposure to 12 μg of LPS). This assessment was supported later by Thorn [100] who assumed that inhalation of 30–40 μg LPS seems to be a threshold level for inducing clinical symptoms and lung function changes in healthy human subjects. The British authors [99] used purified LPS (LPS-PW), which was less appropriate for the study of exposure effects than CE-A. In the conclusion of their work, Rylander et al. [28] expressed an opinion that their results support the hypothesis that bacterial endotoxins cause mill fever, grain handler's fever and humidifier fever.

Based on the above-described experiments, Taylor et al. [101] constructed an exposure chamber in which human subjects could be exposed to study effects of the cotton dust inhalation, using *P. agglomerans* endotoxin as a measurable standard. Using an acetone adhesion process, *P. agglomerans* endotoxin was adsorbed onto respirable cellulose particles to create the endotoxin aerosol (CCP). A dry powder dust generator was refined to consistently disperse small quantities of the aerosol into the chamber to maintain dust concentrations at approximately 250 $\mu g/m^3$. This was an excellent research tool which could be used for precise quantification of the effects of exposure to cotton dust and other organic dusts in conditions highly imitating real exposure. Unfortunately, we have not found further information on the progress of the experiments with the use of this chamber.

The experiments on human subjects were supported by the results obtained on isolated human blood cells acquired from volunteers. Thus, Holt et al. [102] demonstrated that the killed *P. agglomerans* cells stimulate *in vitro* human PMNs to produce a mediator, probably PAF, causing the release of serotonin from rabbit platelets. The authors suggest that this finding may explain platelet aggregation in the pulmonary capillaries of exposed cotton workers, and the release of bronchoconstricting agents causing a reduction in respiratory function.

In conclusion, the experiments on humans and human cells confirmed the results of animal experiments indicating the important role of endotoxin produced mostly by *P. agglomerans* in causing byssinosis and other disorders due to occupational exposure to cotton dust. Since then, no other cotton dust component has been identified as a more important work-related hazard than endotoxin, and Rylander's view on the significance of the occupational risk of exposure to endotoxin in the cotton industry and need for its prevention expressed in 1987 [49] was supported in the years 1990–2015 by other authors, such as Niven and Pickering [103], Lane et al. [104] and Lai and Chistiani [71]. The latter authors expressed an opinion that byssinosis has features of both asthma and chronic obstructive lung disease (COPD), and that the development of persistent airway inflammation and associated airflow obstruction seen in the chronic stage of disease, is caused by reversal in the lung macrophage: dendritic cell ratio due to textile dust-related endotoxin exposure.

Exposure to endotoxin in cotton dust and risk of lung cancer. The question raised by many authors whether exposure to dust-borne endotoxin protects against cancer was not unequivocally solved to-date, but certainly deserves attention in the light of successful treatment of cancer with the use of *P. agglomerans* LPS achieved by Japanese scientists [13, 105, 106], which will be discussed in detail in the last article of this *Pantoea*-series. Henderson and Enterline [107] and Enterline et al. [108] expressed an opinion that exposure to airborne endotoxin decreases cancer mortality in cotton workers. According to the latter authors, endotoxin in cotton dust may inhibit the growth of cancer by stimulating the activity of macrophages, by the release of interferon, by mitogen activity, by the induction of the tumour necrosis factor, or by some other mechanisms. Lange [109] found in the workers of cotton, wood and agricultural industries lower than expected death rates from cancer and suggested, as a protective mechanism, stimulation of the immune system by endotoxin contaminating dusts occurring in these industries. These views have been confirmed by more recent studies. The meta-analysis by Lenters et al. [110], based on high-quality epidemiologic studies, supported the hypothesis that occupational exposure to endotoxin in cotton textile production and agriculture is protective against lung cancer. Similarly, Mc Elvenny et al. [111] proved by an epidemiological follow-up study of British cotton industry workers, that increase in the cumulative exposure to endotoxins in cotton dust significantly reduced the risk of death from lung cancer. Although the postulated protective anti-cancer action of endotoxin in organic dusts is not confined to the *P. agglomerans* LPS, but also applies to LPSs of other Gram-negative bacteria present in these dusts, the abundant occurrence of *P. agglomerans* as a major Gram-negative contaminant of many of these dusts, primarily those of plant origin [25, 112], underlines the important role of this species. The probable role of *P. agglomerans* was supported by the experimental work of Lange and Sykora [113] who demonstrated the reduction of lung cancer progress in mice

injected with Lewis lung carcinoma cells, and exposed later to the inhalation of *P. agglomerans* endotoxin aerosol.

In contrast to the presented study, Christensen et al. [114] in Canada did not find a decreased risk of lung cancer among subjects exposed to cotton and wool dusts. In spite of the fact that the authors did not estimate the cumulative exposure to endotoxin, as carried out, for example, in the study by Mc Elvenny et al. [111], this work indicates that the problem of the reduction of lung cancer by environmental endotoxin produced by *P. agglomerans* and other Gram-negative bacteria remains open and needs further studies.

CONCLUSIONS

The main conclusion of this review is that *Pantoea agglomerans* and the biologically potent endotoxin produced by this bacterium, represent an important occupational risk for the workers of cotton mills, mostly for those engaged in cardrooms, as a potential cause of the acute stage of byssinosis and other respiratory disorders. The bulk of evidence on the role of *P. agglomerans* in causing byssinosis enabled the application of efficient prevention strategies by overall reduction of dust levels, which succeeded in 5–8-fold decrease in the frequency of chest tightness and an over shift drop in $FEV_{1.0}$ among workers, as well as by avoiding of cotton cargoes with a high content of *P. agglomerans* and endotoxin, and setting the appropriate guidelines on permissible concentrations of these adverse factors in the air [115]. However, *P. agglomerans* should not be regarded as the sole pathogen. Ryan et al. [29] mentioned N-formylmethionyl peptides from bacterial cells, cotton plant tannins, and glucans, as other possible adverse components in cotton dust. Moreover, other microbial cell wall agents: lipoteichoic acid and peptidoglycans from Gram-positive bacteria, chitin and (1→3)-β-D-glucan from cell walls of fungi and as yet unknown components of the cotton plant, must be considered as potential non-specific disease agents [58]. Also, with the contemporary methods we are not able to determine which part of endotoxin present in cotton dust is produced by *P. agglomerans,* and which by the other Gram-negative bacteria. We must also keep in mind that in the hitherto conducted research on the etiopathogenesis of byssinosis, the role of bacterial, fungal and plant antigens as potential specific inducers of disease was generally underestimated, and it cannot be excluded that specific immunologic reactions, mostly cellular, may evoke the disease, mainly its chronic stage, similar to that which happens in other diseases caused by exposure to organic dusts [116, 117]. Nevertheless, these remarks do not preclude the major role of *P. agglomerans* endotoxin, and possibly other antigens produced by this bacterium, in causing byssinosis, as well as the need for using the *P. agglomerans* products in further studies on etiopathogenesis of this mysterious disease.

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