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REVIEW ARTICLE

Pantoea agglomerans: a mysterious bacterium of evil and good. Part II – Deleterious effects: Dust-borne endotoxins and allergens – focus on grain dust, other agricultural dusts and wood dust

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Abstract

Pantoea agglomerans, a Gram-negative bacterium developing in a variety of plants as epiphyte or endophyte is particularly common in grain and grain dust, and has been identified by an interdisciplinary group from Lublin, eastern Poland, as a causative agent of work-related diseases associated with exposure to grain dust and other agricultural dusts. The concentration of *P. agglomerans* in grain as well as in the settled grain and flour dust was found to be high, ranging from 10^4–10^8 CFU/g, while in the air polluted with grain or flour dust it ranged from 10^3–10^5 CFU/m^3 and formed 73.2–96% of the total airborne Gram-negative bacteria. The concentration of *P. agglomerans* was also relatively high in the air of the facilities processing herbs and other plant materials, while it was lower in animal farms and in wood processing facilities. *Pantoea agglomerans* produces a biologically-potent endotoxin (cell wall lipopolysaccharide, LPS). The significant part of this endotoxin occurs in dusts in the form of virus-sized globular nanoparticles measuring 10–50 nm that could be described as the 'endotoxin super-macromolecules'. A highly significant relationship was found (R=0.804, P=0.000927) between the concentration of the viable *P. agglomerans* in the air of various agricultural and wood industry settings and the concentration of bacterial endotoxin in the air, as assessed by the *Limulus* test. Although this result may be interfered by the presence of endotoxin produced by other Gram-negative species, it unequivocally suggests the primary role of the *P. agglomerans* endotoxin as an adverse agent in the agricultural working environment, causing toxic pneumonitis (ODTS). Numerous experiments by the inhalation exposure of animals to various extracts of *P. agglomerans* strains isolated from grain dust, including endotoxin isolated with trichloroacetic acid (LPS-TCA), endotoxin nanoparticles isolated in sucrose gradient (VECN), and mixture of proteins and endotoxin obtained by extraction of bacterial mass in saline (CA-S), showed the ability of these extracts to evoke inflammatory and fibrotic changes in the lungs, to stimulate alveolar macrophages to produce superoxide anion (O_2^-), interleukin-1 (IL-1) and chemotactic factors for other macrophages and neutrophils, and to increase the pulmonary concentrations of toll-like receptors and chemokines. The most potent properties showed the CA-S which may be attributed to the allergenic properties of *P. agglomerans* proteins enhanced by the presence of the autologous endotoxin. The results of these experiments are in accord with the clinical studies which revealed a high reactivity of the agricultural and grain industry workers to allergenic extracts of *P. agglomerans*, and the presence in these populations of hypersensitivity pneumonitis and asthma cases caused by this bacterium. *P. agglomerans* has been also identified as a potential causative agent of allergic dermatitis in farmers and of allergic pulmonary disorders in cattle. In conclusion, similar to the cotton industry, also in the grain industry and in agriculture, *Pantoea agglomerans* should be regarded as one of the major causative agents of work-related diseases, caused by the adverse effects of protein allergens and endotoxin produced by this bacterium.

Key words

Pantoea agglomerans, endotoxins, allergens, ultrastructure, agricultural dusts, grain dust, wood dust, agricultural workers, immunologic response, animal experiments, hypersensitivity pneumonitis, toxic pneumonitis, asthma

Abbreviations

BAL = bronchoalveolar lavage; CA-S = cell-derived mix of protein antigens and endotoxin, obtained by the extraction of bacterial mass with saline (0.9% NaCl); CE-A = cell-bound endotoxin, obtained by the precipitation of bacterial mass with acetone (acetone powder); CCP = bacterial cells coupled to cellulose powder; FEV_1 = forced expiratory volume in one second; HP = hypersensitivity pneumonitis; IL-1 = interleukin 1; KCS = killed cells suspension; LCS = live cells suspension; LPS = lipopolysaccharide; LPS-PW = lipopolysaccharide extracted by the phenol/water method; LPS-TCA = lipopolysaccharide obtained by the extraction with trichloroacetic acid and precipitation with acetone; PAM = pulmonary alveolar macrophages; PMN = polymorphonuclear leukocytes; VC = vital capacity; VECN = vesicular endotoxin-containing nanoparticles

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Introduction

Respiratory, skin and general symptoms associated with exposure to grain dust have been known for several centuries among farmers and grain industry workers [1, 2], and

according to the present state of knowledge, include dyspnea, cough, shortness of breath, chest tightness, expectoration, wheezing, irritation of eyes, nose and skin, fever, chills, muscle ache, tiredness and malaise [3, 4, 5]. Subjective symptoms are accompanied by declines in lung function, which could be of both obstructive and restrictive character [5, 6, 7, 8]. Classification of the occupational disorders evoked by grain dust has not been unequivocally unified, but essentially two groups of diseases are distinguished:

a) allergic diseases due to specific sensitization to one or more components of grain dust, followed by the appearance of pathologic symptoms at repeated contact with these component(s);
b) non-specific diseases due to toxic and/or irritant action of various components present in grain dust.

Allergic diseases associated with the exposure to grain dust include: allergic asthma, hypersensitivity pneumonitis (allergic alveolitis, granulomatous pneumonitis), allergic rhinoconjunctivitis, and eczema [3, 9, 10]. A wide spectrum of allergens have been identified as causative agents, comprising fungi, bacteria, mite and insect proteins, and plant allergens originating from grain [3, 4, 9]. Non-specific diseases which may be caused by exposure to grain dust comprise: non-allergic asthma, toxic pneumonitis (organic dust toxic syndrome, ODTS, previously defined as 'grain fever'), chronic obstructive pulmonary disease (COPD, usually a mix of chronic bronchitis and emphysema), pruritus, dermatitis, and mucous membrane irritation (MMI) [4, 6, 10]. Among the common causes of these conditions are reported the constituents of the cell wall of microorganisms developing on grain: primarily endotoxin (LPS) produced by Gram-negative bacteria, and also (1→3)-β-D-glucans produced mostly by fungi, as well as peptidoglycan and lipoteichoic acid (LTA) produced mostly by Gram-positive bacteria [10, 11, 12, 13]. The pathogenic role of mycotoxins, the toxic secondary metabolites of fungi, also cannot be excluded [9].

In contrast to cotton dust, the role of *Pantoea agglomerans* in causing disorders due to inhalation of grain dust, both specific as a source of strong allergens and non-specific as a source of potent endotoxin, has not been sufficiently highlighted until now in the world's literature. Research on this problem has not been sponsored by any potent organization, such as the National Cotton Council in the USA which supported research on cotton dust effects, and has not been the subject of extensive international collaboration. In this situation, most of the research has been carried out by our interdisciplinary group in Lublin, a medium-size city in eastern Poland. For almost 50 years we have been continuing work on all aspects of the afore-mentioned problem which we regard as a very important one [14, 15, 16, 17, 18, 19, 20, 21]. The presented study is a summary of previously performed research on the significance of *P. agglomerans* in causing disorders in the people and farm animals occupationally exposed to grain dust, to other agricultural dusts (such as dusts from herbs), and to wood dust. The research has been performed by a collaborating interdisciplinary group from the Institute of Rural Health, the Medical University, the University of Life Sciences, and the Medical Research Centre of State Railways in Lublin, with the participation of the late Leszek Kuś (1939–1997), Marian Durda, Radosław Śpiewak, Zofia Prażmo, and the authors of the present study in the area of human medicine, and Zbigniew Pomorski and Iwona Taszkun in the area of veterinary medicine. The authors also acknowledge the very significant contribution made to the afore-mentioned problem by other colleagues from Lublin, as well as by prominent scientists from other countries who collaborated with us, among them, the late Robert Burrell (1933–2012), James Tucker, Stephen A. Olenchock, William G. Sorenson, and Daniel M. Lewis from the USA, C. Peter Warren from Canada, Lennart Larsson from Sweden, Marco Chilosi from Italy, Francois Huaux from Belgium, Rolf Ziesche from Austria and Bhawanipant N. Pande from India. Some scientists from the other countries working independently from us, such as Anthony J. De Lucca from the USA and Jillian RM Swan and Brian Crook from the United Kingdom, also contributed significantly to the problem of *P. agglomerans* in agricultural dusts as an important health hazard.

Pantoea agglomerans as a predominant bacterium in grain dust

Pantoea agglomerans is an ubiquitous bacterium widely associated with plants as an epiphytic or endophytic organism [22, 23]. It occurs frequently as an epiphyte on grain, constituting up to 75–99% of the total bacterial biota [24, 25, 26, 27, 28]. *P. agglomerans* is particularly common on freshly harvested grain and is regarded by some agricultural microbiologists as an indicator of the good quality of the grain [25, 28]. The measured concentrations of this bacterium in grain and grain dust are very high (Tab. 1). In grain, they occurred at the levels of 10^4–10^7 CFU/g, whereas in samples of settled grain dust collected in Poland and the USA, they were greater, at the levels of 10^6–10^8 CFU/g [29, 30, 31, 32, 33, 34, 35, 36]. The samples of various species of grain dusts collected in India contained smaller quantities of *P. agglomerans* at the levels of 10^4–10^7 CFU/g, which appeared to be lowest in rice and highest in sorghum [37].

P. agglomerans is a major constituent of the microbiota of grain dust, forming 54.6–92.0% of the Gram-negative bacteria in the samples from Poland and the USA, and 25.0–42.6% in the samples from India. The content of this bacterium in the total microbiota of dust is smaller, ranging from 0.5–69.4% and from 0.04–11.7%, respectively. The concentration of *P. agglomerans* in grain dust may show a marked variation depending on season and conditions of storage. DeLucca and Palmgren [35] observed in samples of grain dust collected from corn stored in two grain elevators along the Mississippi River in Louisiana, that the concentration of *P. agglomerans* revealed a marked season-dependent variation from 30.0×10^3 CFU/g in October to $9{,}417.0\times10^3$ CFU/g in March, whereas percent of this bacterium in Gram-negative biota of grain dust was highest in warm months (April, June, September and November) when it formed 60–87% of all Gram-negative bacteria, and lowest in winter, forming only 10% in January.

After cleaning and grinding of grain in a mill, a drastic decrease in the amount of *P. agglomerans* could be observed, and its measured concentration in flour dust constituted, on average, only 0.05% of the concentration in grain dust [29, 30].

Pantoea agglomerans is also a predominant bacterium in the air of premises contaminated with grain dust, which was evidenced in the studies performed on the territory of eastern Poland. Its highest concentrations, at the level of 10^5 CFU/m^3, were recorded in small ground-floor granaries and grain-cleaning rooms of big mills, while the concentrations at the level of 10^4 CFU/m^3 were found in big elevators, small

Table 1. Presence of *Pantoea agglomerans* in samples of grain as well as settled grain and flour dusts (expressed as concentration per 1 gram), compared to other bacteria

Grain or grain/flour dust [References]	Concentration of *P. agglomerans* in the sample (CFU/g × 10^3) Mean (range)	Average percent of Gram-negative bacteria (%)	Average percent of total microbiota (%)
Wheat (*Triticum vulgare*) grain[a] (Warsaw region, Poland) [36]	620.0[b]	99.0[b]	61.1[b]
Oats (*Avena sativa*) grain + vetch (*Vicia* sp.)[c] (Szczecin region, Poland) [32]	47,820.0[b]	64.5[b]	42.5[b]
Barley (*Hordeum vulgare*) grain[c] (Szczecin region, Poland) [32]	40.0[b]	83.3[b]	0.5[b]
Grain dust[c] (Szczecin region, Poland) [32]	72,170.0 (64,540.0–79,700.0)	81.4 (69.9–92.9)	17.5 (2.3–32.7)
Grain dust (Lublin Region, Poland) [29, 30]*	302,870.0 (23,700.0–769,900.0)	75.0 (34.8–95.0)	49.1 (22.8–75.3)
Grain dust (Lublin Region, Poland) [31]**	5,100.0 (900.0–11,100.0)	85.6 (80.0–91.2)	57.3 (25.7–67.5)
Grain dust (Cracow Region, Poland) [33]	8,700.0 (677.0–22,550.0)	92.0 (84.0–96.1)	69.4 (58.3–86.4)
Grain dust (Louisiana, USA) [34]	15,355.0 (61.3–30,650.0)[d]	61.3	44.4[d]
Grain dust (Louisiana, USA) [35]	4,400.0 (30.0–9,417.0)[d]	54.6 (10.0–87.0)[d]	21.9 (10.0–33.8)[d]
Wheat dust (Aurangabad Region, India) [37]	500.0 (0.0–1,000)	25.0 (0.0–50.0)	11.7 (0–23.4)
Sorghum (*Sorghum vulgare*) dust (Aurangabad Region, India) [37]	18,470.0 (0.0–55,200)	42.6 (0–66.7)	7.0 (0–11.3)
Rice (*Oryza sativa*) dust (Aurangabad Region, India) [37]	50.0 (0.0–100.0)	25.0 (0.0–50.0)	0.04 (0.0–0.08)
Pearl millet (*Pennisetum typhoides*) dust (Aurangabad Region, India) [37]	100.0 (0.0–200.0)	40.0 (0.0–80.0)	0.85 (0.0–1.7)
Flour dust (Lublin Region, Poland) [29, 30]	140.0 (15.0–387.0)	93.3 (46.7–98.5)	15.1 (7.5–22.6)

[a]Used as a substrate for cultivation of mushroom spawn;
[b]Single sample examined;
[c]Sample associated with cases of toxic pneumonitis among students shoveling grain;
[d]Estimates recalculated from original authors' data;
*Results published in 1978;
**Results published in 1986;
N.d. = Not determined.

country mills, at grain threshing on farms, and in some other industrial settings where grain dust is also present, such as feed mixtures plant and malt-house (Tab. 2) [29, 30, 31, 38]. The lowest concentrations of *P. agglomerans*, at the levels of 10^2–10^3 CFU/m^3, were recorded in settings contaminated in flour dust, such as big mills (at milling and sacking of flour and bran), and bakeries where the concentrations of *P. agglomerans*, similar to the case of settled dusts, constituted only a fraction (0.2–1.1%) of those recorded in the air of small granaries massively polluted with grain dust [29, 30, 39]. Small concentrations of *P. agglomerans*, at the level of 10^3 CFU/m^3, were also noted in such industrial settings as a pearl-barley

Table 2. Presence of *Pantoea agglomerans* in air of grain processing premises and related industries (expressed as concentration per 1 m^3), compared to other bacteria

Facility [References]	Concentration of *P. agglomerans* in the air (CFU/m^3 × 10^3) Mean (range)	Percent of the airborne Gram-negative bacteria (%)	Percent of total airborne microbiota (%)
Grain industry, eastern Poland Big grain elevators [29, 30]	52.2 (9.4–422.8)	88.2 (80.8–93.1)	40.4 (21.9–58.2)
Small ground-floor granaries [29, 30]	434.7 (197.6–794.7)	79.7 (70.0–90.1)	33.7 (24.2–53.5)
Big mills: grain-cleaning rooms [29, 30]	109.3[a]	91.9[a]	57.7[a]
Big mills: milling and sacking of flour and bran [29, 30]	4.9 (2.2–7.0)	73.2 (55.0–81.8)	21.8 (13.3–29.7)
Small country mills [29, 30]*	17.9 (6.4–35.8)	73.9 (54.0–83.7)	25.5 (14.7–40.1)
Small country mills [31]**	20.2[a]	79.2[a]	15.9[a]
Grain threshing on farms [38]	84.5 (73.0–96.0)	96.0 (88.9–100)	2.7 (1.3–9.1)
Bakery, cereal and beer industries, eastern Poland Bakery [39]	0.8 (0.2–1.4)	47.1 (28.3–74.7)	4.0 (1.4–7.4)
Feed mixtures plant [29, 30]	16.7 (7.5–65.1)	37.5 (33.8–42.9)	6.0 (4.4–10.9)
Pearl-barley plant [29, 30]	1.7 (0.5–4.0)	29.8 (21.2–60.9)	1.2 (1.1–1.4)
Malt house [29, 30]	51.2 (15.7–102.3)	36.7 (7.9–62.9)	9.4 (1.7–20.7)
Production of mushroom spawn [36]	2.4 (0.0–17.8)	45.3 (9.8–86.8)	0.8 (0.0–29.6)

[a]Samples were taken at a single site;
*Results published in 1978;
**Results published in 1986.

plant and a plant producing mushroom spawn [29, 30, 36]. In all the grain industry settings, *P. agglomerans* was prevalent in the Gram-negative biota and total microbiota of the air, forming 73.2–91.9% and 21.8–57.7% of the total, respectively. In the industries related to grain industry (such as feed mixtures and beer producing facilities), the corresponding figures were 29.8–47.1% and 0.8–9.4%, respectively (Tab. 2). The mean concentrations of *P. agglomerans* alone stated in big elevators, small ground-floor granaries, grain-cleaning rooms of big mills and in a malt house, exceeded the threshold level of 2×10^4 CFU/m^3 proposed by Górny and Dutkiewicz [40] for the total concentration of Gram-negative bacteria in premises contaminated with organic dusts.

The data from Poland on the common occurrence of *P. agglomerans* in the air of occupational environments contaminated with grain dust were confirmed by the studies performed in some other countries. Thus, Swan and Crook [41] reported *P. agglomerans* as one of the predominant bacterial species in samples of airborne grain dust collected on nine farms and two dockside grain terminals in south-east England. In India, Verma and Pathak [42] determined *P. agglomerans* as a dominant bacterium among Enterobacteriaceae isolated from air on the urban grain-market area. According to Lacey [43], *P. agglomerans* commonly colonizes plant surfaces before harvest, but did not appear to be common in British moist grain silos or Canadian grain elevators.

Pantoea agglomerans occurs abundantly in entire working environments polluted with grain dust, including humans and insect pests. The bacterium was found to occur in the upper respiratory tract of the grain elevator and grain mill workers, having been isolated from 24.0% and 7.9% of throat swab cultures, and from 49.3% and 26.2% of nose swab cultures, respectively. The frequency of positive isolations in the more exposed workers of grain elevators was significantly greater than in grain mill workers (P=0.0017 and P=0.0011, respectively) [29, 30].

P. agglomerans has also been isolated from grain weevils (*Sitophilus granarius*), the pest insects collected in a granary. In the sample of whole triturated insects, the bacteria occurred in the concentration of 1.9×10^3 CFU/g, whereas in the sample of triturated intestines the concentration of *P. agglomerans* was much greater and amounted to 366.8×10^3 CFU/g, forming 50.0% of Gram-negative biota and 42.9% of the total microbiota [29, 30]. This finding suggests that the described in literature cases of the occupational respiratory allergy to faecal dust of grain weevils [44, 45, 46] might be due, at least in part, to the presence of the strong allergens of *P. agglomerans*.

Occurrence of *P. agglomerans* in other agricultural dusts, wood dust and other dusts occurring in the working and living environments

Herb processing on farms and in factories. In the samples of settled dusts from marjoram and yarrow herbs collected in big herb processing factories located in eastern Poland, *P. agglomerans* occurred at the relatively high level of 10^6 CFU/g, but formed only a small fraction of Gram-negative flora, which was dominated by *Alcaligenes faecalis* (Tab. 3)

Table 3. Presence of *Pantoea agglomerans* in samples of various plant materials and settled plant dusts, expressed as concentration per 1 gram

Plant material or dust [References]	Concentration of *P. agglomerans* in the sample (CFU/g × 10^3) Mean (range)	Percent of Gram-negative bacteria (%)	Percent of total microbiota (%)
Settled dusts from herbs (eastern Poland)			
Marjoram (*Majorana hortensis*) herb dust [47]	1,700.0[a]	0.02[a]	0.02[a]
Yarrow (*Achillea millefolium*) herb dust [47]	4,700.0[a]	2.6[a]	2.6[a]
Sage (*Salvia officinalis*) herb dust [47]	0.0[a]	0.0[a]	0.0[a]
Birch (*Betula verrucosa*) leaves dust [47]	0.0[a]	0.0[a]	0.0[a]
Calamus (*Acorus calamus*) rhizome dust [47]	304.5[a]	77.4[a]	58.5[a]
Settled plant dusts (Aurangabad region, India)			
Green gram (*Phaseolus arrus*) dust [37]	425.0 (150–700)	52.9 (12.5–93.3)	18.8 (0.14 -37.4)
Red gram (*Cajanus cajan*) dust [37]	100.0[a]	100[a]	23.8[a]
Amaranth (*Amaranthus spinosus*) dust [37]	1,450.0[a]	100[a]	3.9[a]
Tobacco			
Stored tobacco leaves (eastern Poland) [51]	54.1 (0.0–405.0)	57.7 (15.4–100)	12.1 (0–72.8)
Wood and wood dust			
Timber logs: sapwood of American basswood (*Tilia americana*), West Virginia, USA [60]	300.0[a]	100[a]	3.6[a]
Wood chips and settled dust in paper factories (northern Poland) [64]	216.4 (0.0–1,320.0)	23.0 (0.0–53.8)	N.d.
Settled wood dust in a sawmill processing beech wood (southeastern Poland) [61]	3,800.0 (1,100.0–6,500.0)	10.0 (5.0–15.0)	N.d.
Settled wood dust in a factory producing furniture from beech wood (southeastern Poland) [61]	2.9 (0.7–5.0)	100	N.d.
Pollen surface (southern Poland)			
Birch (*Betula verrucosa*) [68]	12.1 (0.0–33.0)[b]	100	N.d.
Alder (*Alnus glutinosa*) [68]	11.5[a]	100[a]	N.d.
Hazel (*Corylus avellana*) [68]	0.0[a]	0.0[a]	N.d.
Mugwort (*Artemisia vulgaris*) [68]	2.3[a]	90.0[a]	N.d.
Rye (*Secale cereale*) [68]	5.5[a]	100[a]	N.d.

[a]Single sample examined;
[b]Estimate recalculated from original authors' data;
N.d. = Not determined.

Table 4. Presence of *Pantoea agglomerans* in the air of various agricultural and industrial settings (expressed as concentration per 1 m^3), compared to other bacteria

Facility [References]	Concentration of *P. agglomerans* in the air ($CFU/m^3 \times 10^3$) Mean (range)	Percent of airborne Gram-negative bacteria (%)	Percent of total airborne microbiota (%)
Herb processing on farms (eastern Poland)			
Peppermint (*Mentha piperita*) processing [48]	268.5 (160.2–509.0)	50.8 (41.6–68.0)	11.9 (8.5–22.0)
Chamomile (*Matricaria recutita*) processing [48]	11.6 (0.0–52.0)	92.2 (83.2–100)	12.2 (0.0–68.2)
Valerian (*Valeriana officinalis*) roots processing [49]	1.7 (0.0–6.9)	13.7 (0.3–99.4)	1.1 (0.0–15.1)
Thyme (*Thymus vulgaris*) processing [50]	1.3 (0.3–2.1)	20.0 (10.5–57.1)	0.4 (0.2–1.1%)
Processing of other plant materials (eastern Poland)			
Processing of hop (*Humulus lupulus*) cones on farms [53]	0.8 (0.0–11.9)	50.0 (40.0–60.0)	4.6 (0.0–6.2)
Horticulture seeds storage [54]	3.4 (0.0–7.5)	21.3 (9.1–27.9)	0.1 (0.0–3.6)
Flax (*Linum usitatissimum*) scutching on farms [52]	21.9 (6.1–42.5)	43.1 (13.3–71.0)	5.4 (0.8–10.0)
Haymaking [38]	39.3 (4.6–74.0)	75.0 (41.0–84.4)	10.8 (4.2–16.2)
Animal houses (eastern Poland)			
Cowsheds [55]	0.4 (0.0–0.8)	9.8 (0.0–11.2)	0.2 (0.0–0.4)
Horse stables [56]	2.3 (0.0–6.2)	35.9 (0.0–92.5)	1.6 (0.0–5.5)
Piggeries [57]	2.7 (0.5–5.7)	12.2 (3.7–16.8)	12.2 (3.7–16.8)
Wood processing industry			
Paper factories (northern Poland) [64]	0.02 (0.0–0.08)	17.8 (0.0–90.4)	N.d.
Factory producing furniture from beech wood (southeastern Poland) [61]	0.0013 (0.0–0.006)	100	N.d.

N.d. = Not determined.

[47]. In the settled dust from calamus rhizome, *P. agglomerans* occurred at the level of 10^5 CFU/g and prevailed in the Gram-negative and total microbiota, but was absent in the dust from sage herb and birch leaves (Tab. 3) [47].

At the processing of herbs, cultivated for medicinal use on farms in eastern Poland, large quantities of *Pantoea agglomerans*, at the levels of 10^4 and 10^5 CFU/m^3, were released into air during camomile and peppermint processing, respectively (Tab. 4) [48], exceeding, in the case of peppermint, the threshold level proposed by Górny and Dutkiewicz [40] for total Gram-negative bacteria. This species dominated among the airborne Gram-negative bacteria, forming 50.8–92.2% of the total count. Smaller airborne concentrations of *P. agglomerans*, at the level of 10^3 CFU/m^3 and 13.7–20.0% of the total Gram-negative count, were recorded during valerian roots and thyme processing (Tab. 4) [49, 50]. In two big herb processing factories located in eastern Poland, *P. agglomerans* occurred generally in smaller quantities and was isolated from the air only at a half (7 out of 14) of the working stands processing various kinds of herbs with the maximal concentration below 2×10^5 CFU/m^3 [47].

Handling of other plant materials. The presence of *P. agglomerans* was evidenced in dusts from three species of plants cultivated in India where it occurred at the levels of 10^5–10^6 CFU/g (Tab. 3) [37]. This species was dominant among Gram-negative bacteria forming 52.9–100% of the total count, whereas its frequency in the total microbiota was distinctly lower, accounting for 3.9–23.8% of the total. *P. agglomerans* occurred at the level of 10^4 CFU/g on tobacco leaves stored in a factory (in the form of air-dried leaves, leaves after curing and leaves after curing and over 1-year storage), forming 57.7% of Gram-negative flora and 12.1% of the total microbiota (Tab. 3) [51]. *P. agglomerans* appeared to be a common air pollutant during flax scutching on farms and at haymaking, where it occurred at the level of 10^4 CFU/m^3, exceeding the threshold level proposed by Górny and Dutkiewicz [40] for total Gram-negative bacteria, and forming respectively 43.1% and 75.0% of the Gram-negative biota [38, 52]. Its concentrations were smaller during the processing of hop cones on farms and in a horticulture seed store, where it occurred, respectively, at the levels of 10^2 CFU/m^3 and 10^3 CFU/m^3, forming 50.0% and 21.3% of the total Gram-negative count [53, 54].

Animal houses. *Pantoea agglomerans* occurs in the air of animal houses at the rather low mean levels of 10^2–10^3 CFU/m^3 (Tab. 4) which, however, may rapidly increase during feeding of animals with plant fodders. On average, the species forms 9.8–35.9% of Gram-negative biota and 0.2–12.2% of the total airborne microbiota in animal houses which is usually dominated by Gram-positive bacteria (Tab. 4) [55, 56, 57, 58]. Ławniczek-Wałczyk et al. [59] have isolated *Pantoea* spp. from the air of poultry houses heavily contaminated with bacteria.

Wood and wood industry premises. *P. agglomerans* has been consistently recovered from timber logs stored in forests or in sawmill yards, but usually in quantities much lower compared to agricultural plants. A relatively large concentration of this bacterium was found in the sapwood of the American basswood (*Tilia americana*) in the USA where it occurred at the level of 10^5 CFU/g, and was the only constituent of Gram-negative biota [60]. In six various species of timber logs examined in south-eastern Poland, the quantities *P. agglomerans* were much lower and therefore are shown rather as the frequencies of positive isolations than the concentrations in CFU/g. *P. agglomerans* occurred mostly in the sapwood of examined logs, having been isolated from 6.7–13.3% of examined wood samples with estimated maximal concentrations ranging from below 1×10^1 to below 1×10^4 CFU/g, than in the heartwood, where it was found in 0–10.0% of the samples, with estimated maximal

Table 5. Presence of *Pantoea agglomerans* in samples of wood from timber logs stored in forests or sawmill yards and in the air of wood industry premises, expressed as frequency of isolation (percent of positive samples out of total examined)

Kind of sample [References]	Frequency of isolation (positive/examined samples, percent)	Maximal concentration in positive samples
Samples from timber logs, south-eastern Poland		
Scots pine (*Pinus sylvestris*), sapwood [61, 62, 63]	2/20 (10.0%)	$<1 \times 10^2$ CFU/g
Scots pine (*Pinus sylvestris*), heartwood [62, 62]	0/20 (0.0)	0.0
Norway spruce (*Picea abies*), sapwood [62]	4/30 (13.3%)	$<5 \times 10^3$ CFU/g
Norway spruce (*Picea abies*), heartwood [62]	0/30 (0.0)	0.0
Silver fir (*Abies alba*), sapwood [62]	2/30 (6.7%)	$<1 \times 10^4$ CFU/g
Silver fir (*Abies alba*) heartwood [62]	1/30 (3.3%)	$<1 \times 10^3$ CFU/g
English oak (*Quercus robur*), sapwood [62]	3/30 (10.0%)	$<1 \times 10^1$ CFU/g
English oak (*Quercus robur*), heartwood [62]	0/30 (0.0)	0.0
Birch (*Betula verrucosa*), heartwood [61, 62]	3/30 (10.0%)	$<1 \times 10^1$ CFU/g
Beech (*Fagus sylvatica*), sapwood [61, 62, 63]	3/30 (10.0%)	$<6 \times 10^2$ CFU/g
Air samples		
Sawmills processing coniferous wood, eastern Poland [65]	3/7 (42.9%)	$<8 \times 10^3$ CFU/m^3
Sawmills processing deciduous wood, eastern Poland [65]	0/8 (0.0)	0.0
Sawmills processing pine wood, southeastern Poland [61, 63]	4/20 (20.0%)	$<2 \times 10^2$ CFU/m^3
Sawmills processing beech wood, southeastern Poland [61, 63]	2/25 (8.0%)	$<1 \times 10^4$ CFU/m^3
Fiberboard and chipboard factories, eastern Poland [66]	3/14 (21.4%)	$<4 \times 10^3$ CFU/m^3

concentrations ranging from below 0.0 to below 1×10^1 CFU/g (Tab. 5) [61, 62, 63].

In the examined wood processing facilities producing furniture and paper, *P. agglomerans* has been recovered from the air by Prażmo et al. [61, 64] at the low levels of 10^0–10^1 CFU/m^3 (Tab. 4). The frequency of *P. agglomerans* isolation from the air was greater in the sawmills processing coniferous wood, ranging from 20.0–42.9%, than in sawmills processing deciduous wood, where it ranged from 0–8.0% (Tab. 5) [61, 63, 65]. In the fibreboard and chipboard factories, this bacterium was recovered, on average, from 21.4% of air samples (Tab. 5) [66]. Ławniczek-Wałczyk et al. [67] isolated *Pantoea* spp. also from the air of a power plant combusting forest and agricultural biomass (such as wood chips) together with coal. The concentrations of *P. agglomerans* in the samples of settled wood dust and wood chips collected in wood industry premises were at the levels of 10^3–10^6 CFU/g, forming 10.0–100% of Gram-negative flora (Tab. 3) [61, 64]. Generally, in the airborne or settled wood dust, *P. agglomerans* was usually less numerous compared to *Rahnella aquatilis* and *Rahnella* spp. strains, which proved to be dominant Gram-negative bacteria and the most important source of endotoxin in this environment [62, 65].

Pollen grains. Śpiewak et al. [68] determined the concentration of Gram-negative bacteria and endotoxin on the surface of five kinds of pollen grains, known as the common causative agents of the seasonal, allergic rhinoconjunctivitis (pollinosis) in Poland [69]. The concentration of Gram-negative bacteria varied from $0–12.1\times10^3$ CFU/g and *P. agglomerans* was the only species recovered, except for one colony of *Acinetobacter* cultured from one sample (Tab. 3). The concentration of endotoxin varied from 7.7–37.5 μg/g and was not significantly correlated with the concentration of *P. agglomerans* [69]. On the basis of the obtained results, it cannot be excluded that in particular cases endotoxin and/or allergens of *P. agglomerans* may aggravate the allergic reactions caused by pollen.

House dust and aerosols in dwellings. *P. agglomerans* has been isolated from 10.0% of the settled house dust samples collected from beds on the territory of the Upper Silesian conurbation, Poland, with maximal concentration below 2×10^4 CFU/g [70]. The frequency of its isolation was the same as that of *Escherichia coli* and *Acinetobacter calcoaceticus*, but greater compared to thirteen other species of Gram-negative bacteria. The average concentration of endotoxin in the examined samples was, on average, 80.0 μg/g. The results indicate that *P. agglomerans* is only one of many components of a rich Gram-negative biota of house dust, and thereof the allergens and endotoxin produced by this bacterium are probably of limited significance in causing disorders related to house dust exposure. Such a conclusion is in accordance with the results of Górny [71] and Górny and Dutkiewicz [40] who found, also on the territory of the Upper Silesian conurbation, the presence of *P. agglomerans* in the air of only 10.0% of examined dwellings with the maximal concentration below 10^2 CFU/m^3.

Endotoxin of *P. agglomerans* as a potential cause of respiratory disorders due to inhalation of grain dust and the other agricultural dusts

The hypothesis on the possible role of endotoxins produced by Gram-negative bacteria associated with vegetable dusts in causing work-related respiratory and general symptoms was raised for the first time with relation to cotton dust [72, 73, 74] which has been discussed in the former article of *Pantoea*-series [23]. Pernis et al. [73] presumed that 'grain fever' described in silo workers may be evoked by endotoxin. This presumption was strengthened in 1976 by Dutkiewicz [14] who demonstrated for the first time a potent biological activity of endotoxins isolated from *P. agglomerans*, using strains isolated from airborne grain dust and other sources. He isolated also for the first time from a sample of the settled grain dust a substance revealing endotoxin properties.

Table 6. Comparison of concentrations of viable *Pantoea agglomerans* (CFU/m^3) and endotoxin (ng/m^3) in air of various agricultural and wood processing working environments in Poland

Working environment	Concentration of airborne *Pantoea agglomerans* (CFU/m^3)	Concentration of airborne endotoxin (ng/m^3)
Threshing grain [38]	84,500.0	18,150.0
Crushing grain in a mill [31]	20,200.0	54,900.0
Grain elevators [29, 88]	52,200.0	234,000.0
Haymaking [38]	39,300.0	17,970.0
Threshing flax [52]	21,900.0	30,000.0
Peppermint processing [48]	268,500.0	312,500.0
Chamomile processing [48]	11,600.0	3,130.0
Valerian processing [49]	1,700	33,430.0
Hop processing [38]	800.0	52.2
Cowsheds [55, 94]	400.0	9.25
Horse stables [56, 94]	2,300.0	26.1
Piggeries [57, 94]	2,700.0	31,250.0
Paper factories [64]	20.0	21.0

Dutkiewicz concluded that endotoxins produced by *P. agglomerans* and other Gram-negative bacteria associated with grain dust should be considered as a potential cause of work-related disorders among workers exposed to the inhalation of grain dust and related dusts of plant origin [14, 23]. A later study by Dutkiewicz et al. [75] demonstrated that *P. agglomerans* endotoxin associated with grain dust revealed more potent biological activities compared to endotoxins produced by other species of bacteria occurring in organic dusts.

The structural backbone of the *P. agglomerans* endotoxin was found to be similar to other Gram-negative species, but some distinguished features were also described. Tsukioka et al. [76], seeking the structural peculiarities which could explain the very high activity in the stimulation of macrophages revealed by *P. agglomerans* LPS-PW endotoxin, determined the structure of lipid A of this preparation, and established that the *P. agglomerans* LPS is constructed with at least two kinds of lipid A of different levels of acylation. One is of the same type as that of *Escherichia coli* with hexa-acyl lipid A, and the other is the *Salmonella minnesota* type with hepta-acyl lipid A. These results are essentially in accord with the studies by Cole et al. [77] and Boué and Cole [78] who found in *P. agglomerans* LPS the existence of at least two lipid A types, which differed only in the presence of an additional oxygen atom. The lower molecular weight compound is very similar to the well-characterized lipid A from *Salmonella minnesota*. Although the biological activity of *P. agglomerans* LPS is mostly related to the structure of lipid A, some authors also ascribed an important role to the structure of O-specific polysaccharide as a determinant of this activity. Karamanos et al. [79] found that O-specific polysaccharide of the LPS-PW isolated from the standard strain of *P. agglomerans* consists of the pentasaccharide repeating units containing fucose, rhamnose, and glucose. By contrast, Cimmino et al. [80] analyzed the structure of O-specific polysaccharide of *P. agglomerans* LPS-PW isolated from the strain developing in olive, and determined that it consists of the linear tetrasaccharide repeating units containing D-rhamnose as the major constituent and minor quantities of glucose and galactose. Recently, Shimada et al. [81] expressed an opinion that the modulation of macrophage activity by LPSp, the *P. agglomerans* LPS-PW used in Japan for treatment of various diseases, is possibly mediated by as yet unknown or uncharacterized receptor(s), including lectins, present in the O-specific polysaccharide.

The presence of bacterial endotoxin in the settled grain dust was confirmed in 1978 by Olenchock et al. [82] who, using the newly-introduced *Limulus* test, found a relatively low concentration of endotoxin at the level of 10^2 ng/g. In subsequent years, much greater quantities of endotoxin in the samples of settled grain dust were detected, at the levels of 10^5 ng/g [31]. In parallel, in the air of many agricultural working environments polluted with organic dusts, high levels of bacterial endotoxin were detected, creating a significant hazard for exposed workers. On average, the highest quantities at the levels of 10^2–10^5 ng/m^3, were recorded during grain handling in various facilities in the USA [83, 84], Finland [85, 86], Poland [31, 87, 88], Norway [89], and The Netherlands [90], during handling of grass seeds in The Netherlands and Denmark [91, 92], and during herb processing, flax threshing and raising pigs on Polish farms [48, 49, 52, 87, 88]. In some other agricultural working environments, such as tobacco processing, the animal feed industry, cattle farms, potato processing, pearl barley processing, and hop growing and processing, the levels of the airborne endotoxin were distinctly lower, being equal to 10^0–10^1 ng/m^3 [53, 85, 86, 87, 88, 93, 94] (results reported in EU (Endotoxin Units) were converted to nanograms assuming 1 ng = 10 EU).

To-date, no commonly used species-specific method for endotoxin determination exists which would allow for the identification of its origin, e.g. bacterial species which produced endotoxin detected in dust. The *Limulus* test detects only endotoxin, which could be produced by a multitude of the species of Gram-negative bacteria, and hence it is not possible to determine which part of the airborne endotoxin mentioned above was produced by *P. agglomerans*, as except for Poland, the species composition of airborne Gram-negative biota was not determined along with the endotoxin. Nevertheless, comparison of the concentrations of viable *P. agglomerans* and endotoxin detected by the *Limulus* test in various agricultural and wood processing working environments in Poland (Tab. 6), shows a highly significant correlation (R=0.804, P=0.000927) between these variables (Fig. 1) [29, 31, 38, 48, 49, 52, 53, 55, 56, 57, 64,

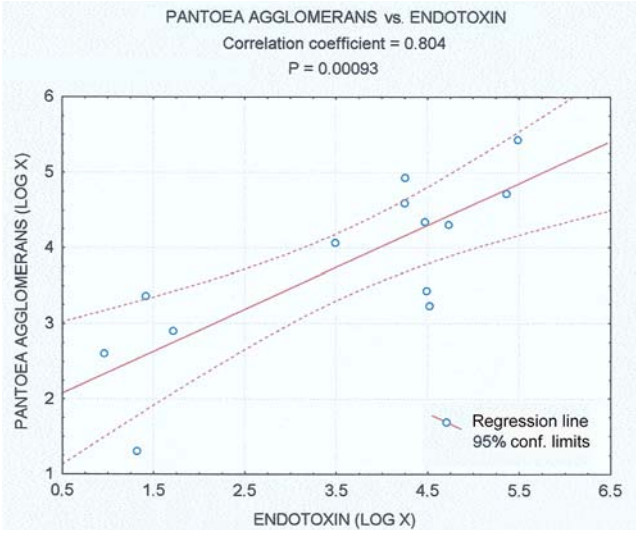

Figure 1. Correlation between concentration of culturable *Pantoea agglomerans* (CFU/m^3, $\log_{10}$ x) and endotoxin (ng/m^3, $\log_{10}$ x) in the air of various agricultural and wood processing working environments in Poland

88, 94]. Although this comparison has some limitations (a total endotoxin was determined instead of a species-specific, in one case the measurements of *P. agglomerans* and endotoxin concentrations were not parallel) the regression line distinctly demonstrates the overall tendency of an increase in the airborne endotoxin content, together with the content of *P. agglomerans*, mostly at the processing of grain, herbs, and flax which, of course, does nor preclude the possibly important role of other bacterial species as endotoxin producers in the agricultural environment.

Studies performed by researchers at the Iowa center in the USA confirmed the importance of endotoxin as a principal component of grain dust causing work-related symptoms. In a study of 410 grain workers, Schwartz et al. [95] demonstrated that the prevalence of work-related symptoms (cough, phlegm, wheezing, chest tightness, dyspnea) in the workers, decrease in respiratory function (FEV_1, $FEF_{25–75}$, FEV_1/FVC), and increase in bronchial reactivity (20% decline in FEV_1 in response to inhaled histamine) were strongly correlated with the concentrations of bacterial endotoxin to which the workers were exposed, but not with the concentrations of total dust, similarly as shown earlier by Donham et al. [96] in swine confinement buildings and by Smid et al. [93] in the animal feed industry. The authors concluded that endotoxin appears to play a primary role in the development of airway symptoms and airflow obstruction among grain workers. As an experimental complementation of this epidemiological study, Jagielo et al. [11] subjected fourteen healthy volunteers to the inhalation of corn dust extract and *Escherichia coli* lipopolysaccharide (LPS) solution, and found a high degree of similarity in responses to both factors, manifested by the occurrence of similar respiratory symptoms (chest tightness, cough, dyspnea, and sputum production), acute declines in FEV_1, and increases in bronchoalveolar lavage inflammatory cells and mediators. In the opinion of the authors of the current study, the significance of these interesting studies would probably be higher if they were supported by the bacteriological examinations of grain dust, which most probably would allow for identification of the dominant bacterial species (*P. agglomerans* or another species of Gram-negative bacteria) representing the main source of adverse endotoxin in the tested grain dust samples [95], and would enable the use of LPS of this species in the inhalation challenge instead of *E. coli* LPS which occurs extremely rarely in grain dust [11]. Such an attitude would be similar to that applied by many experienced researchers who, in their experiments on the effects of endotoxin in cotton dust had used the *P. agglomerans* LPS as being dominant in this environment [97, 98, 99, 100], and would certainly make the afore-mentioned results more reliable for science and for prevention.

The important role of endotoxin in causing disorders related to respiratory exposure to grain dust is also underscored by other scientists [10, 101, 102]. The most common disorder caused by the exposure to endotoxin present in grain dust and other agricultural dusts is toxic pneumonitis (Organic Dust Toxic Syndrome – ODTS), previously often referred to as 'grain fever'. The disorder is characterized by dyspnea, chest tightness, cough, shivering, fever, malaise and other respiratory and general symptoms. As this condition is non-specific and could also be elicited by other components of grain dust (peptidoglycan, fungal spores etc.), the establishing of the causal relationship in individual cases could be difficult, and the number of ODTS cases which could be ascribed to *P. agglomerans* as a cause is rather low. Such a case has been reported by Salkinoja-Salonen et al. [103] who described the case of a man who developed chills, slight fever, dyspnea, and malaise after washing bean sprouts at a commercial growing process. Medical examination showed a decrease in the transfer factor, transient increase of white cell count in blood, but no radiographic changes. *P. agglomerans*, occurring in large numbers on bean sprouts and in the air of the work environment was recognized as the cause of the illness. Most probably it was a case of ODTS caused by the endotoxin of *P. agglomerans*, although the possibility of specific allergic reaction cannot be definitely excluded. Endotoxin produced by *P. agglomerans* could also be suspected as one of causative agents of the collective cases of toxic pneumonitis noted in 1979 in groups of high school pupils shoveling grain on State farms in north-western Poland [32]. The bacterium was found to occur at a high level of 10^7 cfu/g in 2 out of 10 samples of grain and grain dust associated with the disease (Tab. 1), but in other samples fungi and actinomycetes prevailed which were recognized as the potential causative agents of similar importance.

Potential role of *P. agglomerans* endotoxin in causing disorders due to inhalation of tobacco smoke

The distinct (over 55%) prevalence of *P. agglomerans* among the Gram-negative bacteria isolated from tobacco leaves determined by Larsson et al. [51] (Tab. 3) is very meaningful in the light of studies which evidenced a significant increase of endotoxin content in rooms polluted with tobacco smoke. Already in 1998, Górny and Dutkiewicz [104] demonstrated in the aerobiological studies conducted in the Upper Silesian conurbation in Poland, that the concentrations of airborne endotoxins determined with the *Limulus* (LAL) test in apartments polluted with tobacco smoke were greater compared to those not polluted in all size ranges of particulate aerosol (dust). The highest level of endotoxins was found in the fraction of fine particles below 5 μm. In the following year, Hasday et al. [105], also using the *Limulus* test, found high concentrations of endotoxin (LPS) in cigarette

tobacco, amounting to 17.8–26.8 μg per cigarette, and in airborne particles of tobacco smoke generated from the cigarettes by an automatic smoking machine, equal to 45.1–121.0 ng/cigarette in mainstream and 17.5–74.9 ng/cigarette in side-stream. The authors concluded that the estimated delivered LPS dose from smoking one pack of cigarettes per day (2,400 ng) is comparable to the levels of LPS associated with adverse health effects in cotton textile workers. They suggested that the bioactive LPS in cigarette smoke may contribute to the pathogenesis of chronic bronchitis that develops in susceptible cigarette smokers.

Of significance is a study by Larsson et al. [106] who determined the concentration of endotoxins in the air by using the gas chromatography-tandem mass spectrometry (GC-MSMS) method for determining of 3-hydroxy fatty acids (3-OH FAs), the chemical markers of LPS. This method is superior to the *Limulus* test, as it enables biochemical characteristics of endotoxins. The authors stated that tobacco smoking dramatically increases the endotoxin content in the air, and expressed the opinion that the endotoxin present in cigarettes contributes to the high prevalence of respiratory disorders among smokers. They demonstrated that the concentration of LPS in the air of a room polluted with tobacco was 12.1 pmol/m^3, being 120 times higher than in smoke-free indoor air. The amount of LPS inhaled during active smoking equaled 17.4 pmol per cigarette. The LPS associated with tobacco smoke was characterized by a strong predominance of 3-OH FAs of fourteen carbon chain lengths (3-OH $C_{14:0}$). This is noteworthy in the light of the studies performed recently by Varbanets et al. [107] who analyzed the fatty acid composition of *Pantoea agglomerans* LPS, and found that in all the studied strains, 3-OH $C_{14:0}$ was the predominant component (31.7% – 39.1%, depending on the strain). The prevalence of the 3-OH $C_{14:0}$ fatty acids in lipid A of *P. agglomerans* was also reported in an earlier work by Tsukioka et al. [76]. Thus, comparison of the above-cited studies provides a strong indication that a considerable part of endotoxin present in tobacco smoke is produced by *Pantoea agglomerans*. This confirms the results of study by Larsson et al., cited earlier [51], in which the large quantities of *P. agglomerans* and its prevalence in Gram-negative flora of stored tobacco leaves was associated with considerable amounts of endotoxin (LPS), ranging from 5.48–18.8 pmol/mg.

The significant exposure to endotoxin associated with tobacco smoke has been confirmed by further studies of the Larsson's group, who demonstrated that smoking is associated with high exposure to endotoxin which may contribute to inflammation and airway disease in active and passive smokers. Sebastian et al. [108] found that the air concentrations of endotoxin were 4–63 times higher in the rooms of students who smoked than in identical rooms of non-smoking students. Larsson et al. [109] stated that the LPS concentration in tobacco ranged from 4,820–11,320 pmol per cigarette, whereas in the tobacco smoke it ranged from 823–1,504 pmol/cigarette in mainstream and from 41–71 pmol/cigarette in side-stream. Szponar et al. [110] evidenced that the mean air concentrations of LPS were significantly higher in smoking than in non-smoking rooms in studied private houses (0.0017 vs. 0.0007 nmol/m^3), and at worksites (0.0231 vs. 0.0006 nmol/m^3). 3-OH $C_{14:0}$ was the main 3-OH FA, which again indicates *P. agglomerans* naturally colonizing tobacco leaves as a possible source of endotoxin in tobacco smoke.

Hazardous nanoparticles – ultrastructure of *P. agglomerans* endotoxin in organic dusts

In 1982, the Finnish researchers Lounatmaa and Helander [111] observed in broth cultures of *P. agglomerans*, *Klebsiella oxytoca* and *Pseudomonas putida* strains isolated from cotton, peeling-off the outer membrane in the form of either ribbons or round vesicles measuring 8–12 nm in diameter. In subsequent years, Dutkiewicz et al. [75, 112] showed that similar vesicular, spherical structures measuring 30–50 nm and having a characteristic trilaminar or 'triple-tracked' (dark-light-dark) membrane, corresponding to the structure of the outer membrane of bacteria, are formed by *P. agglomerans* cells not only in the liquid media, but also on the solid agar media, often by convolution of the outer membrane (Fig. 2) or by a budding-like process (Fig. 3). These vesicular, spherical (discoid, globular) structures, produced most often in the deteriorating or dividing cells, were almost identical to those observed in the thin-sections of the isolated LPS-PW preparation of *P. agglomerans* (Fig. 4). Their identity with the bacterial LPS has been definitely confirmed by immuno-staining with the anti-LPS rabbit antibodies or

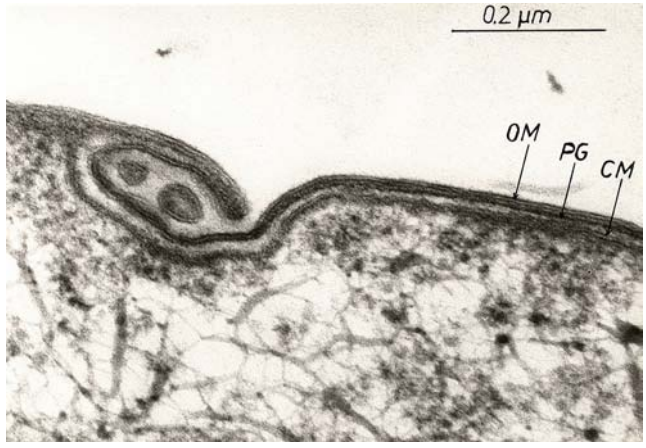

Figure 2. Thin section of the cell envelope of *Pantoea agglomerans* (strain M-10-3, agar culture), clearly showing three layers: outer membrane (OM), peptidoglycan layer (PG) and cytoplasmic membrane (CM). The OM has a very distinct trilaminar structure. Inside a pouch created by convolution of the cell envelope, two membrane vesicles (VECN) are seen, showing morphology identical with that of thin-sectioned LPS particles (Fig. 4). According to Dutkiewicz et al. [112]

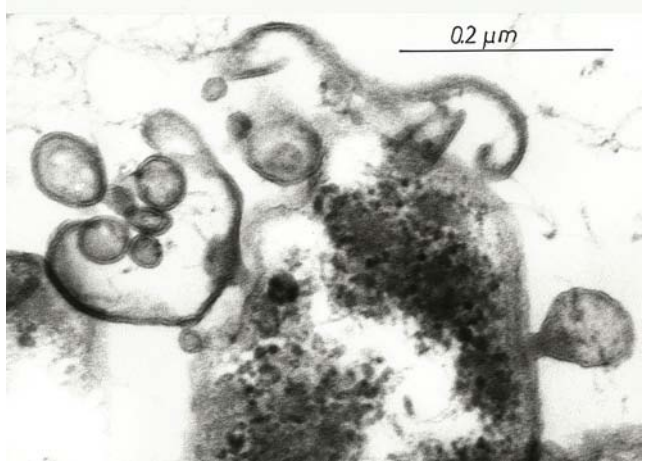

Figure 3. Thin-sectioned cell of *Pantoea agglomerans* (strain WD-72, agar culture) showing formation of vesicles (VECN) from outer membrane by a budding-like process. Note structures suggesting formation of smaller blebs inside larger ones by internal budding. According to Dutkiewicz et al. [112]

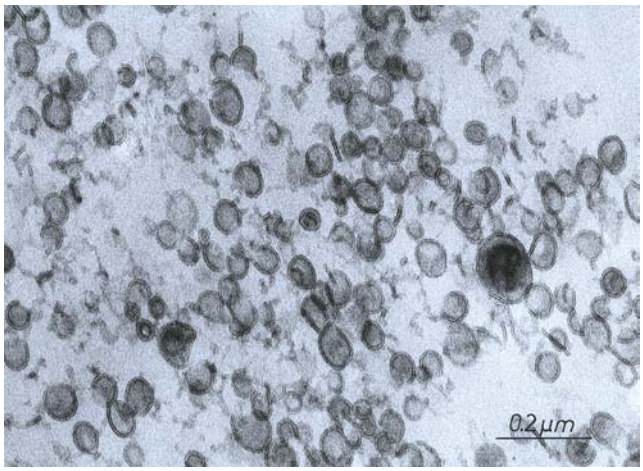

Figure 4. Thin-sectioned particles of the solated lipopolysaccharide (LPS-PW) from *Pantoea agglomerans* (strain M-10-3). According to Dutkiewicz et al. [112]

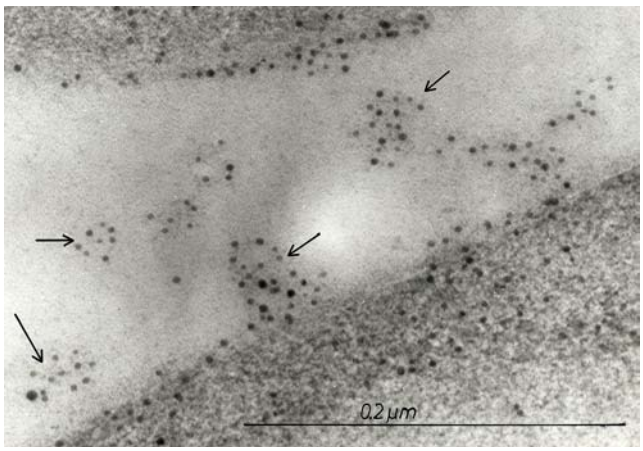

Figure 5. Thin-sectioned cells of *Pantoea agglomerans* (strain WD-72, agar culture) immunostained with rabbit antiserum against LPS of *P. agglomerans* and gold-labeled with anti-rabbit IgG. Arrows show aggregations of gold particles upon structures corresponding in shape and size to vesicles budding from outer membrane. According to Dutkiewicz et al. [112]

anti-lipid A mouse antibodies, followed by labeling with immunogold conjugated with the anti-rabbit or anti-mouse IgG (Fig. 5).

As the next step, the presence of vesicular endotoxin-containing nanoparticles (VECN) of *P. agglomerans* has been demonstrated for the first time in the natural environment, using as a medium thin-sectioned sawdust sample taken from the sapwood of American basswood (*Tilia americana*), which was found earlier by culture and the *Limulus* test to contain viable *P. agglomerans* cells and endotoxin [112, 113]. By electron microscopy, small VECN measuring 10–20 nm were found peeling off the outer membranes of bacteria. After detachment, they increased in size up to 30–50 nm (Fig. 6). Similar to the culture, the identity of these structures with *P. agglomerans* LPS was confirmed by the specific immunogold labelling (Fig. 7). In the following years, similar VECN structures were found in other organic dusts containing *P. agglomerans*, such as grain dust [87] (Fig. 8). It was also demonstrated that VECN could most probably be produced by some other Gram-negative bacteria present in organic dusts, such *Pseudomonas* spp. colonizing the moist heartwood of birch, known as wetwood [114]. Generally, the release of outer membrane vesicles by Gram-negative bacteria has been described by many authors,

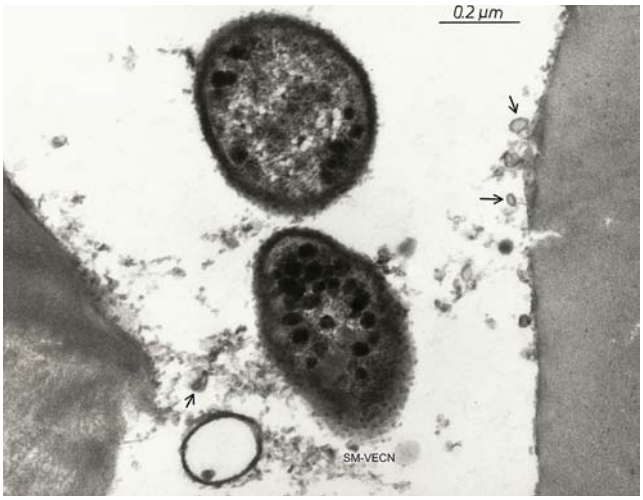

Figure 6. Thin-sectioned sample of pulverized wood from American basswood (*Tilia americana*) showing two big structures corresponding to cells of *P. agglomerans* with small membrane vesicles (marked SM-VECN) peeling-off outer membrane of the lower structure and numerous greater membrane vesicles (marked with arrows) in the lumen of a wood cell. According to Dutkiewicz et al. [112]

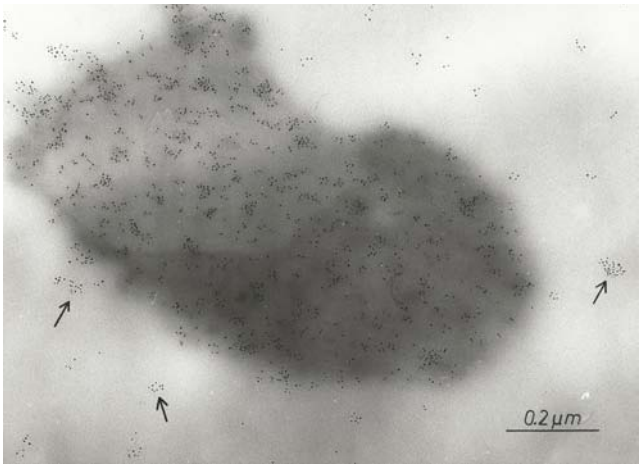

Figure 7. Thin-sectioned sample of pulverized wood from American basswood (*Tilia americana*) immunostained with rabbit antiserum against LPS of *P. agglomerans* and gold-labeled with anti-rabbit IgG. Structure corresponding to the cell of *P. agglomerans* can be seen, stained positively with immunogold. Arrows outside the cell show aggregations of gold particles to smaller structures corresponding in shape and size to membrane vesicles (VECN). According to Dutkiewicz et al. [112]

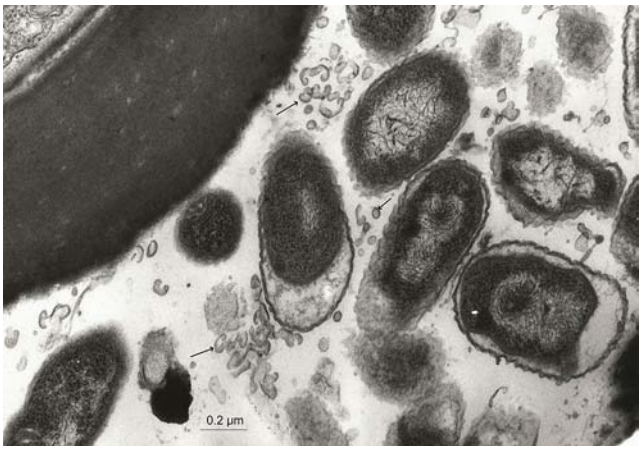

Figure 8. Thin-sectioned sample of grain threshing dust. Numerous vesicular endotoxin-containing nanoparticles (VECN, some marked with arrows) are seen between bigger structures corresponding to cells of *P. agglomerans*. According to Dutkiewicz et al. [87]

though some misleading opinions were expressed. For example, Kulp and Kuehn [115] in their review article on outer membrane vesicles overestimate the role of proteins and underestimate the role of LPS. The cited authors, who surprisingly have not provided any electron micrograph to support their conclusions, seem to have forgotten that LPS is the main component of the outer membrane, and that one of the important functions of the outer membrane vesicles is dissemination of the environmental endotoxin. Proteins may be present in VECN and add an allergenic effect to endotoxin; nevertheless, their quantity is smaller compared to LPS.

The presented study demonstrates that a significant part of environmental 'dust-borne' endotoxin occurs in the form of very small, virus-sized globules measuring 10–50 nm that could be described as the 'endotoxin supermacromolecules'. Even though many of these particles occur in aggregates, the bulk of finely dispersed VECN fraction may easily penetrate into the deep parts of lungs and interact with alveolar macrophages and other immune cells, thus presenting a potential risk of respiratory disorder in workers exposed to the inhalation of organic dusts contaminated with *P. agglomerans* or some other Gram-negative bacteria. Determining of the VECN concentration in the contaminated air needs the use of specialized methods, and so far its value remains unknown, although according to a rough estimate, it could be of the order of 10^9 particles in 1 m^3 of the air.

The current study ascertained that the VECN fraction could be separated from *P. agglomerans* bacterial mass in sucrose density gradients [113]. The experimental works, which will be described in the next section, revealed its potent immunostimulative properties [116, 117].

Experiments proving the potential role of *P. agglomerans* endotoxin and/or allergens in causing grain dust-related disorders

Introduction. The results of the experiments on the effects of the preparations from *P. agglomerans* strains isolated from grain on vertebrate organisms and tissues, performed by the Lublin group from 1980 until recently, show basic similarities to those achieved by researchers studying the effects of the preparations from *P. agglomerans* strains isolated from cotton in the last quarter of the 20th century and described in a previous article of *Pantoea*-series [23], although some essential differences also occured. When experiments with the use of cotton strains recorded in the majority of cases the acute non-specific effects caused by the purified LPS-PW endotoxin, the experiments with the use of grain strains, mainly the strain M-10–3 isolated from the airborne grain dust, were performed mostly using the saline extract (CA-S) of *P. agglomerans* bacterial mass containing proteins, sugars, DNA and RNA (42.3%, 15.2%, 0.018%, and 0.014%, respectively, as determined by spectrophotometric analysis) [Lemieszek MK, unpublished results], with a relatively small quantity of endotoxin (1% of active endotoxin, as assessed by *Limulus* test) [118, 119], and were largely focused on the chronic effects which could be attributed to allergy as the result of a long-term exposure. The use of CA-S is in accord with the results obtained by Rylander [120] and Rylander et al. [99] who found that the effects of cell-bound endotoxin (CE-A) on vertebrate organisms were stronger compared to the purified LPS-PW preparation. However, the experimental model aimed at reproducing the allergenic effects of *P. agglomerans* has not been applied by Rylander or by the majority of researchers working on cotton strains. This may be explained by the fact that byssinosis and other disorders caused by exposure to cotton dust were essentially not related to allergy, whereas those caused by exposure to grain dust were, as demonstrated by numerous cases of hypersensitivity pneumonitis (HP), diagnosed in Polish agricultural workers after exposure to grain dust containing *P. agglomerans* [16, 19].

Effects of the cell-derived mix of protein antigens and endotoxin (CA-S), obtained by the extraction of bacterial mass of *P. agglomerans* with saline. CA-S obtained from the *P. agglomerans* M-10-3 strain isolated from airborne grain dust elicited significant effects *in vitro* on the cultures of pulmonary alveolar macrophages (PAMs) of guinea pig. Milanowski and Dutkiewicz [119] demonstrated with the use of the chemiluminescence method that the *P. agglomerans* CA-S and the extract of *Saccharopolyspora rectivirgula,* stimulated *in vitro* guinea pig PAMs to significantly increase the generation of superoxide anion (O_2^-), which could be additionally augmented by the presence of the complement. These finding suggests the possible participation of toxic oxygen radicals in the pathogenesis of diseases due to the inhalation of grain dust.

Milanowski [121], Milanowski and Dutkiewicz [122] and Milanowski et al. [123, 124] examined in four comparative studies on guinea pig PAMs cultures the stimulating effects of seven agents associated with organic dusts (*P. agglomerans* CA-S and LPS-TCA, *Thermoactinomyces vulgaris* and *Aspergillus fumigatus* cell extracts, protease produced by *Bacillus thermoproteolyticus rocco* and glucans produced by bakers' yeast (*Saccharomyces cerevisiae*) and barley (*Hordeum vulgare*), and of the standard *Escherichia coli* endotoxin on the generation of superoxide anion (O_2^-) by PAMs [121, 122], stimulation of PAMs to kill bacteria [121, 122], induction of interleukin 1 (IL-1) production by PAMs [121, 123] and chemoattractant activities for PAMs and neutrophils [121, 124]. *Pantoea agglomerans* CA-S and LPS-TCA, and *Thermoactinomyces vulgaris* and *Aspergillus fumigatus* cell extracts showed the greatest potency in the stimulation of PAMs to release superoxide anion [122]. Both *P. agglomerans* preparations, *A. fumigatus* cell extract and bakers' yeast glucan revealed the strongest stimulating effect on bactericidal activity of PAMs toward *Staphylococcus aureus*. Of the 8 examined agents, only both *P. agglomerans* preparations and *Th. vulgaris* extract induced strongly the IL-1 production by PAMs [123]. *Escherichia coli* endotoxin showed a significantly weaker effect which corroborates the earlier results achieved by Lewis [125], whereas *A. fumigatus* extract, protease and glucans did not exert any effect at all.

Both *P. agglomerans* preparations and bakers' yeast glucan also showed a strong direct chemoattractant activity, attracting significantly larger numbers of guinea pig PAMs and human neutrophils than other agents. The examined agents also revealed an indirect chemoattractant activity, by stimulating PAMs to release chemotactic factors for other PAMs and for neutrophils [124]. The ability of *P. agglomerans* CA-S and LPS-TCA to stimulate PAMs to release chemotactic factors for other PAMs was weaker compared to *A. fumigatus* extract, protease and bakers' yeast glucan, whereas the ability to stimulate these cells to release chemotactic factors for neutrophils was again very potent, together with *Th. vulgaris* and *A. fumigatus* extracts, and bakers' yeast glucans.

The experiments performed *in vitro* on guinea pigs PAMs by Milanowski et al. [121, 122, 123, 124] clearly showed that *Pantoea agglomerans* protein and lipopolysaccharide components are among the most important agents causing inflammatory reactions after exposure to grain dust and other organic dusts.

The results achieved *in vitro* were confirmed by the experiments performed on guinea pigs *in vivo*. Dutkiewicz and Kuś [126] exposed guinea pigs to a 60 min inhalation of CA-S from the *P. agglomerans* strain M-10-3, and found significant increments of free lung cells in the bronchoalveolar lavage, similar to the results obtained by Rylander and Lundholm [127] who used LCS of *P. agglomerans*. The only difference concerned the ratio of macrophages to lymphocytes, which increased to 2:1 instead of circa 1:1 noted in the control group exposed to saline and in the cited reference [127].

Milanowski [128, 129] subjected guinea pigs to the inhalation challenge with the aerosols of seven of the aforementioned agents associated with organic dusts and of *E. coli*, the same which were used for assessment of the effects *in vitro* [121, 122, 123, 124]. Aerosols of *P. agglomerans* CA-S and LPS-TCA were generated from the solutions made in saline at the concentration of 10 μg/ml, mimicking real exposure in facilities heavily contaminated with grain dust. The challenge with *P. agglomerans* CA-S resulted in a significant increase in the breathing rate of animals by 62%. BAL of exposed animals revealed significantly increased yields of total cells, alveolar macrophages, neutrophils, lymphocytes, and red blood cells eighteen hours post-exposure. The increments were greatest in animals exposed to *P. agglomerans* CA-S and *A. fumigatus* extract. PAMs harvested by BAL showed a significant increase in superoxide anion (O_2^-) production, mostly in animals exposed to *P. agglomerans* CA-S, *A. fumigatus* extract and bakers' yeast glucan. Alveolar macrophages from animals exposed to *P. agglomerans* CA-S showed distinct morphological changes, from smooth and rounded cells to irregular cells with rough surfaces, blebs and filopodia, as shown in the previous study [23].

Kuś [130] and Kuś et al. [17] subjected guinea pigs to a long-term inhalation (up to 50 exposures in 80 days) of CA-S from *P. agglomerans* strain M-10–3 isolated from grain. In histopathologic examinations of the lungs of the exposed animals by light microscopy, the authors observed a progressively developing multifocal interstitial inflammatory lesions, granulomas, and fibrosis within interalveolar septa. These observations were confirmed by ultrastructural examination of the lungs by transmission electron microscopy. After 1–2 exposures, degeneration of endothelial cells and type II pneumocytes was seen, which conforms with the results of Lantz et al. [97] presenting effects of the endotoxin derived from *P. agglomerans* strain isolated from cotton, indicating endothelial cells as a primary target of this endotoxin. Beginning with ten exposures, advanced infiltration of interalveolar septa was observed with neutrophils, eosinophils, lymphocytes, and fairly numerous plasma cells (Fig. 9). In plasma cells, the canals of rough endoplasmic reticulum were dilated and flocculent material was present in their lumen, suggesting the intensified production of immunoglobulins (Fig. 10). In

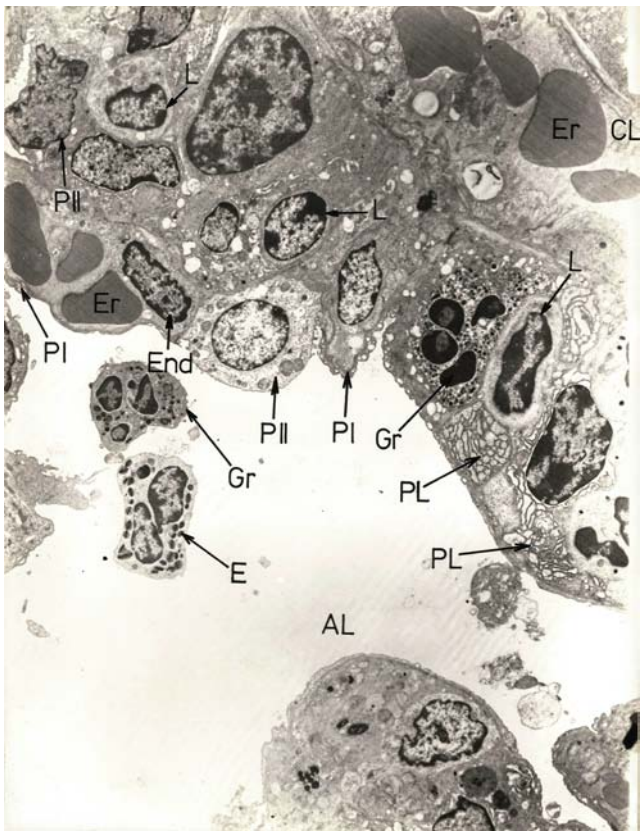

Figure 9. Ultrastructure of the lungs of guinea pigs after 20 exposures to *P. agglomerans* CA-S. Abundant inflammatory cells in interalveolar septa and in the lumen of alveolus: granulocytes (Gr), lymphocytes (L), eosinophils (E), and plasma cells (PL). AL – lumen of alveolus, CL – lumen of capillary, P I – type I pneumocyte, P II – type II pneumocyte, Er – erythrocyte, End – endothelial cell. Electron microscope (EM), × 10,000. According to Kuś et al. [17]

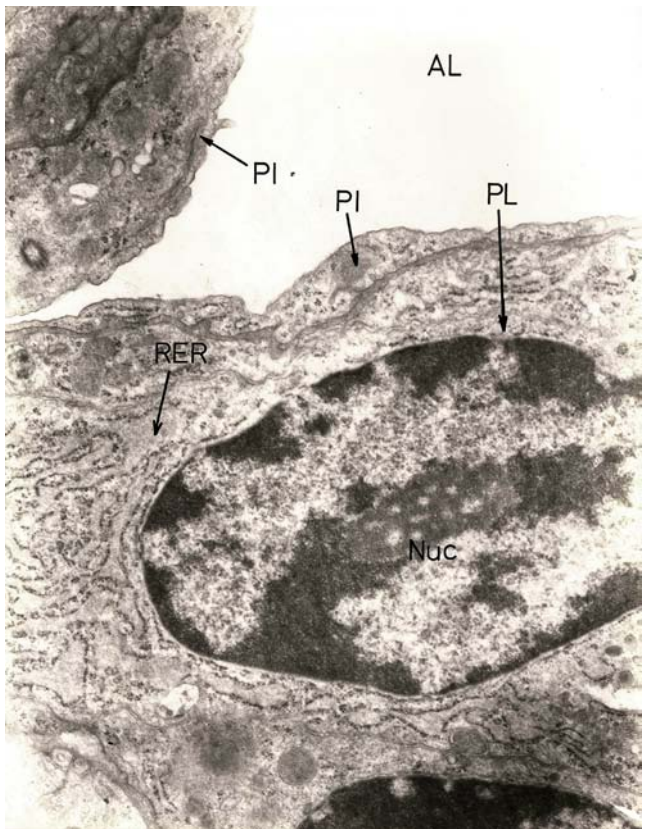

Figure 10. Ultrastructure of the lungs of guinea pigs after 20 exposures to *P. agglomerans* CA-S. Plasma cell (PL) in interalveolar septum. Dilated canals of rough endoplasmic epithelium (RER) in the plasmocyte are filled with flocculent material. Nuc – cellular nucleus, PI – type I pneumocyte, AL – lumen of alveolus. EM, × 36,000. According to Kuś et al. [17]

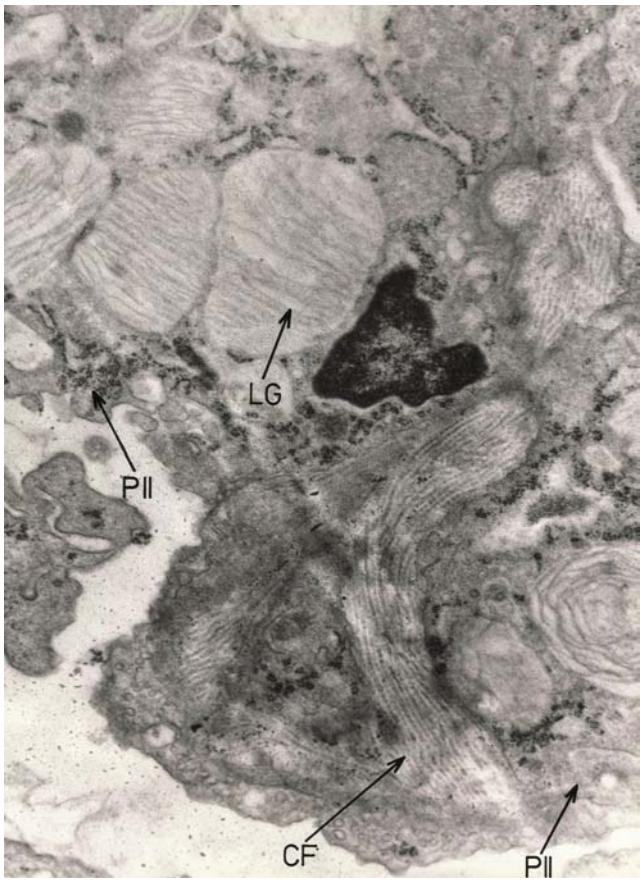

Figure 11. Ultrastructure of the lungs of guinea pigs after 50 exposures to *P. agglomerans* CA-S. Two type II pneumocytes (PII) with numerous lamellar granules (LG) are seen. Between pneumocytes a streak of collagenic fibers (CF) can be seen. EM, × 16,000. According to Kuś et al. [17]

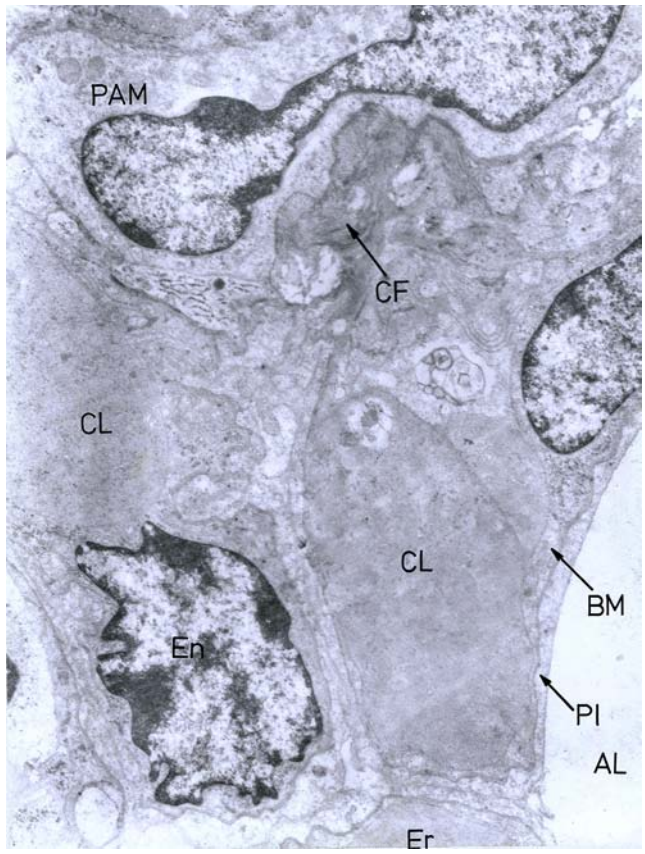

Figure 12. Ultrastructure of the lungs of guinea pigs after 20 exposures to *P. agglomerans* CA-S. Alveolar macrophage (PAM) with clearly deformed nucleus migrating through interalveolar septum. CF – collagenic fibers, BM – capillary basement membrane, CL – lumen of capillary, AL – lumen of alveolus, PI – type I pneumocyte, En – endothelial cell, Er – erythrocyte. EM, × 20,000. According to Kuś et al. [17]

interalveolar septa, the presence of thick bunches of collagenic fibres, of type II pneumocytes containing large amounts of lamellar granules (Fig. 11), and of an increased number of macrophages, occasionally replacing the pneumocytes, was noted. The shape of some macrophages suggested their passage through the alveolar wall (Fig. 12) [17, 130].

Recently, Lemieszek et al. [20] reported the results of a comparative study aimed at reproducing the clinical picture of HP in the mice of the strain C57BL/6J prone to fibrosis, after twenty-eight daily exposures to the inhalation of aerosols of each of the 5 components of organic dusts associated with the etiopathogenesis of HP: *P. agglomerans* CA-S, *P. agglomerans* VECN, extract of *Saccharopolyspora rectivirgula*, extract of *Aspergillus fumigatus*, and extract of dust from grain overgrown with *S. rectivirgula* and *Thermoactinomyces vulgaris*. Lung samples were collected from mice exposed for seven and twenty-eight days, and examined by digitalized histopathology and biochemistry for the presence of inflammatory changes and fibrosis. *P. agglomerans* CA-S appeared to be the sole antigen which evoked a statistically significant fibrosis, and a significant increase of hydroxyproline in the mice exposed for twenty-eight days to this antigen, compared to the control mice exposed to solvent. *P. agglomerans* CA-S also evoked the strongest and statistically significant inflammatory response in the lungs of mice, both after seven and twenty-eight days of exposure. After seven days, significant inflammatory changes were also found in mice exposed to *A. fumigatus* extract, and after twenty-eight days in mice exposed to all antigens. The authors concluded that the *P. agglomerans* CA-S proved to be the best inducer of the pathologic changes attributed to HP, and hence should be used as a triggering agent in the experimental models of this disease. This conclusion is in line with the afore-mentioned results by Kuś et al. [17] who recorded profound histopathological and ultrastructural changes in the lungs of animals subjected to long-term inhalation exposure to the same CA-S preparation of *P. agglomerans*.

The authors successfully applied the proposed model in the following study on the dependence of HP changes on age [131]. It was demonstrated that in 18-month-old mice exposed to *P. agglomerans* CA-S aerosol, a pronounced inflammatory reaction and significant signs of fibrosis were seen already after seven daily exposures, whereas in younger, 3-month-old mice, only inconsiderable inflammatory response and no fibrosis occurred at that time (Fig. 13). After twenty-eight daily exposures, the intensity of pathologic changes was similar in both age groups (Fig. 13). Reported alterations may suggest that older people may develop HP after a relatively shorter period of exposure to harmful antigen than younger individuals, while longitudinal exposure may result in similar pathological changes in both age groups. Lemieszek et al. [131] also proved that the exposure of mice to *P. agglomerans* CA-S aerosol caused an age-dependant increase in lung tissue concentration of both pro-inflammatory (TNFa, IFNγ, IL1a, IL6, IL12, IL17, IL22, CCL3, CCL4, RANTES) and anti-inflammatory cytokines (IL10, TGFβ1).

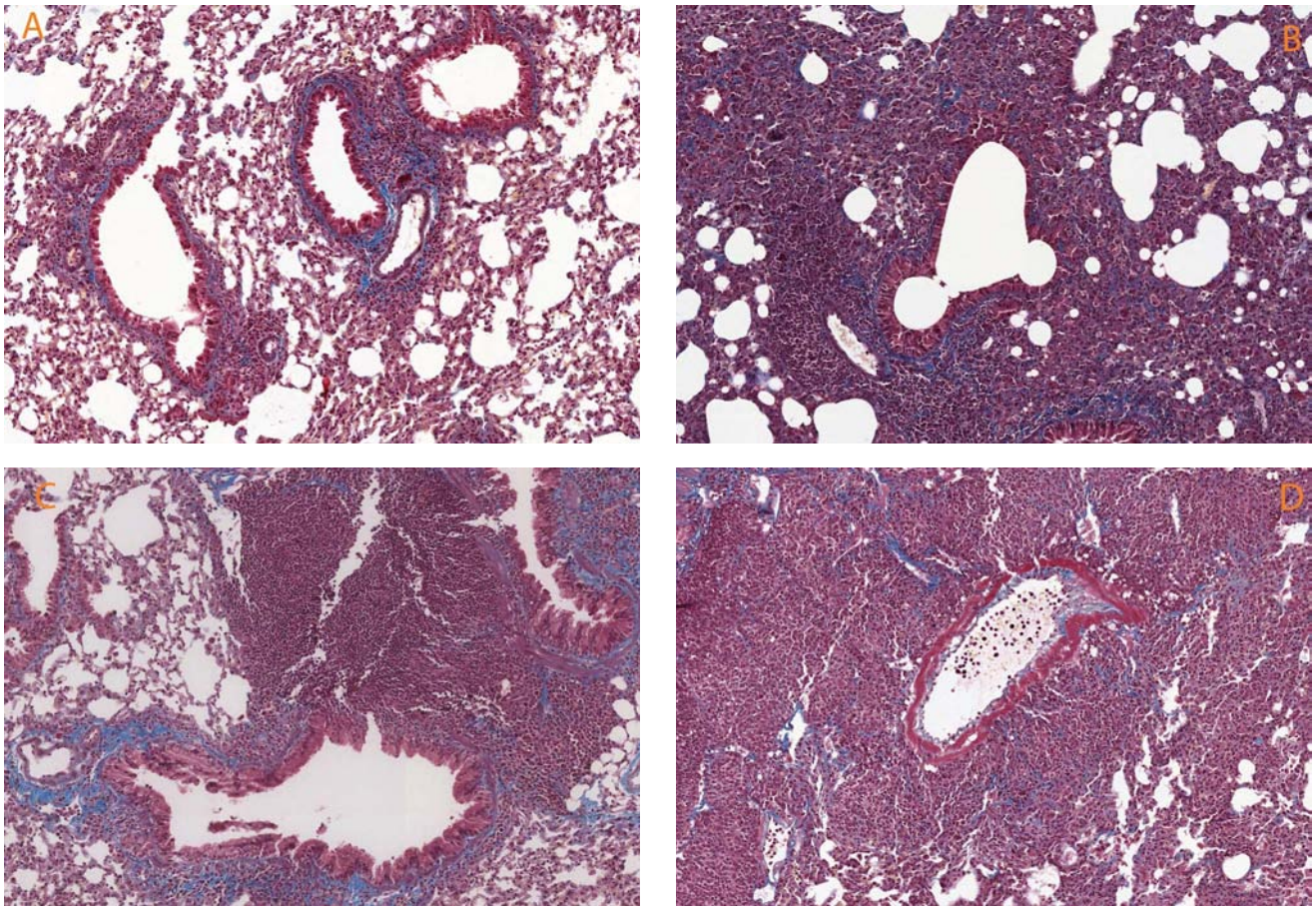

Figure 13. Histopathology examination of the lungs after Masson trichrome staining from mice exposed to inhalation of *P.agglomerans* CA-S. Upper row – results in 3-month-old mice after 7 days (A) and 28 days (B) of exposure. Lower row – results in 18-month-old mice after 7 days (C) and 28 days (D) of exposure. Note lack of fibrosis in young mice after 7 days and advanced interstitial and peribronchial fibrosis after 28 days, whereas in old mice the significant signs of fibrosis were seen already after 7 days and the advanced fibrosis occurred after 28 days. Phot. MK Lemieszek.

Golec et al. [21] investigated changes in the pulmonary levels of some important components of innate immunity, such as peptides, toll-like receptors and chemokines in the course of experimental HP in the 3-month-old and 18-month-old mice, induced by long-term inhalation exposure to *P. agglomerans* CA-S. They noted a significant decrease in the concentration of CRAMP (cathelicidin related antimicrobial peptide precursor) which was more pronounced in older animals. Decrease in the content of cathelicidin, the pleiotropic peptide ensuring pulmonary homeostasis, neutralizing LPS [132] and revealing bactericidal activity toward *P. agglomerans* [133], may impair lung tissue repair and accelerate fibrosis. In contrast, pulmonary concentrations of other investigated components of innate immunity, including the peptide laminin-1-a, toll-like receptors TLR2, TLR4 and TLR8, and chemokines CXCL9 and CXCL10, increased in the course of the experimental HP induced by *P. agglomerans* CA-S, indicating involvement of these components in the pathologic process.

Effects of endotoxins (LPSs) of *P. agglomerans* obtained by extraction of bacterial mass of *P. agglomerans* with trichloroacetic acid (LPS-TCA) or with phenol-water (LPS-PW). In the *in vitro* experiments, *P. agglomerans* LPS-TCA strongly stimulated guinea pigs PAMs to generate superoxide anion, bactericidal activity, IL-1 production, and to direct and indirect chemoattractant activity at a degree comparable to *P. agglomerans* CA-S [121, 123, 124]. By contrast, in the *in vivo* experiments by inhalation challenge, the effects of *P. agglomerans* LPS-TCA on increase in breathing rate, increments of BAL cells, and enhancement of the generation of superoxide anion by BAL cells were smaller compared to those noted after exposure to *P. agglomerans* CA-S [128]. Generally, the presented results confirmed again the suggested important role of *P. agglomerans* endotoxin in eliciting the inflammatory reactions in lungs after inhalation exposure to organic dusts, although the effects of the protein component of this bacterium seem significant and should not be underestimated.

Effects of the vesicular endotoxin-containing nanoparticles (VECN) of *P. agglomerans*. VECN fraction isolated from the *P. agglomerans* strain M-10–3 isolated from grain dust, corresponding morphologically to endotoxin present in organic dusts, showed potent immunostimulative properties. In an acute inhalation experiment, it caused an abundant influx of free alveolar cells into the lungs of guinea pigs, significantly greater compared to control saline and, in the case of lymphocytes and neutrophils, also significantly greater compared to LPS-PW of *P. agglomerans* ($P<0.01$) [116]. Rabbits exposed to long-term inhalation of *P. agglomerans* VECN showed a big increase in the content of circulatory cytokines: total interferon (IFN), interleukin-1 a (IL-1a), and tumour necrosis factor (TNF-a). The rise was highly significant compared to animals that were inhaled with saline. Rabbits exposed to VECN also developed humoral and cellular immunity to *P. agglomerans*, as assessed by the presence of specific precipitins and positive test for the inhibition of leukocyte migration ($P<0.001$) [116]. No

significant difference in phagocytic activity of granulocytes was noted between the rabbits exposed to VECN and controls. The VECN fraction proved to be non-toxic for mice, and its activity in the *Limulus* test was 20 times smaller compared to LPS-PW [116]. The stated in the rabbit model stimulation of cytokine content by VECN has been confirmed *in vitro* in the culture of the human peripheral blood mononuclear cells, where significant increases of IFN-γ and TNF-a content were found [117]. In mice exposed to long-term inhalation of VECN, significant inflammatory changes appeared, but not fibrosis, after 28 exposures [20].

The results of the afore-mentioned experiments may be interpreted from two viewpoints: on the one hand, they indicated strong pro-inflammatory properties of *P. agglomerans* VECN, which creates a risk of pulmonary inflammation in workers exposed to dusts containing VECN supermolecules, but on the other hand, they suggest the possibility of the enhancement of anti-tumour defence mechanisms in exposed individuals, which could explain the lower cancer morbidity in agricultural workers reported by some authors [134, 135], and create the possibility of using non-toxic VECN preparations of *P. agglomerans* as immunostimulants in the treatment of cancer [117].

Concluding remarks. Generally, the strongest adverse properties in the performed experiments showed that the *P. agglomerans* CA-S reproduced in animals the main features of HP, and appeared to be the most potent agent among various bacterial, actinomycetal and fungal factors associated with the etiopathogenesis of HP. This result may be attributed to the potent allergenic properties of *P. agglomerans* proteins enhanced by the presence of autologous endotoxin. Seemingly, in this model, a feedback stimulation may occur in which endotoxin, known for its adjuvant properties, augments the effects of the allergenic non-atopic reactions to protein antigens while, on the other hand, the effects of allergenic reactions increase the impact of endotoxin. Nevertheless, this hypothesis needs experimental confirmation.

Immune response of the exposed occupational populations to allergens of *P. agglomerans*

High immunologic response to *P. agglomerans* in exposed grain workers and farmers. A high reactivity of individuals exposed to grain dust to the antigens of *P. agglomerans* was demonstrated for the first time by Dutkiewicz [15, 30]. Grain store workers and grain farmers exposed to the inhalation of large quantities of viable *Pantoea agglomerans* bacteria (Tab. 2), at the levels of 10^4–10^5 CFU/m^3, reacted in the applied serological and skin tests to CA-S allergen of *P. agglomerans* with a high frequency, significantly greater compared to the reference group of healthy urban dwellers (Tab. 7) [15, 30, 136]. The incidence of positive skin reactions to *P. agglomerans* was significantly greater in the more exposed grain store workers than in less exposed flour handling millers (P<0.001) [15, 30]. This dependence was also found in the complement fixation test (P<0.001), but not in the precipitin test. Flax farmers, hop growing farmers, farmers growing various kinds of herbs, and farmers breeding cattle and pigs, showed mostly a significantly higher frequency of positive reactions to *P. agglomerans* compared to the reference group in the tests for precipitins and inhibition of leukocyte migration, but not in the skin prick test (Tab. 7) [137, 138, 139, 140, 141].

Grain workers reacted also in the precipitin test to lipopolysaccharide antigen (LPS-TCA) of *P. agglomerans* with the frequency of 15.4%, significantly greater (P<0.05) than in the reference group, where the incidence of positive reactions was 2.2% [15, 30].

High incidence of positive precipitin reactions to *P. agglomerans* CA-S was found in two independently examined groups (I and II) of rural inhabitants performing various occupations (35.3% and 35.7%, respectively) (Tab. 7). Rural inhabitants of group I (from eastern Poland and Slovakia) reporting the occurrence of symptoms (dyspnea, cough, fever) after exposure to dust, showed significantly greater prevalence of positive reactions than those without symptoms (46.8% vs. 29.2%; P<0.001) [15, 30].

Similarly, rural inhabitants of group II (from eastern Poland) with diagnosed chronic pulmonary disease, and those with the radiographic abnormalities in the lungs, showed the presence of anti-*P. agglomerans* antibodies significantly more frequently than healthy subjects (respectively, 57.5% vs. 29.6%; P<0.01, and 55.6% vs. 32.3%; P<0.05) [16, 130].

High frequencies of the skin, precipitin and complement fixation reactions with *P. agglomerans* CA-S were found among patients with chronic respiratory disease of various etiology (Tab. 7) [15, 16, 30, 130]. In the group of agricultural workers from Slovakia with different chronic pulmonary diseases, the frequencies of positive precipitin and complement fixation reactions were 49.3% and 60.8%, respectively (Tab. 7) [15, 30, 142, 143]. In another group of rural and urban respiratory patients from eastern Poland, the frequencies of the positive precipitin and complement fixation reactions were 25.0% and 23.8%, respectively [16, 131]. Within this group, the prevalence of the positive precipitin reactions in the subgroup of agricultural workers was significantly greater compared to the subgroup of patients not exposed to organic dusts (31.3% vs. 19.3%; P<0.001) [16, 130].

Greater incidence of positive immunologic reactions to *P. agglomerans* in grain workers and farmers with work-related symptoms. Grain workers reporting symptoms (cough, dyspnea, fever) associated with exposure to grain dust reacted significantly more frequently than asymptomatic ones in skin test (61.8% vs. 42.7%; P<0.001), and in the precipitin test with the *P. agglomerans* CA-S (32.5% vs. 22.5%; P<0.05) and LPS-TCA (22.5% vs. 4.05%; P<0.05) [15, 30]. Similarly, among grain farmers a significant correlation (P<0.05) was found between the occurrence of work-related symptoms or chronic bronchitis and positive precipitin reaction to *P. agglomerans* [136]. Thus, although a positive result of the immunologic test shows merely sensitization, but not a disease, the above-quoted results suggest that the probability of respiratory disease is greater in individuals reacting positively to the antigen of *P. agglomerans*. This is in accordance with the results of Durda et al. [144] who found that grain workers reporting the occurrence of respiratory symptoms (dyspnea, chest tightness, cough), after exposure to grain dust lasting several hours, reacted significantly more often in the inhalation challenge with the diluted CA-S allergen of *P. agglomerans* than those who were asymptomatic (16.1% vs. 6.1%; P<0.05). The proportion of individuals reacting positively in this test showed a significant correlation with work duration (R=0.937; P<0.05), from 5.3% in the group working for less than 5 years to 16.7% in the group working for more than 20 years (mean for all workers = 10.4%). The authors concluded that inhalation challenge with

Table 7. Immune response to allergen of *P. agglomerans* (CA-S) in various populations. Each field shows total positive/total examined (percent)

Immunologic test Occupational population	Skin test (early reactions)	Precipitin test (double diffusion)	Complement fixation test	Test for inhibition of leukocyte migration (MIF)
Grain store workers [15, 30]	48/84 (56.0%)[a***]	26/102 (25.5%)***	28/73 (38.4%)***	N.d.
Grain mill workers [15, 30]	38/194 (19.6%)[a**]	53/211 (25.1%)***	19/126 (15.1%)*	N.d.
Rural inhabitants performing various occupations – Group I [15, 30]	18/120 (15.0%)[a]	194/550 (35.3%)***	7/77 (9.1%)	N.d.
Rural inhabitants performing various occupations – Group II [16, 131]	N.d.	65/182 (35.7%)***	35/65 (53.8%)***	N.d.
Grain farmers [137]	17/76 (22.4%)[b**]	25/76 (32.9%)***	N.d.	N.d.
Flax growing farmers [138]	4/51 (7.8%)[b]	29/51 (56.9%)***	N.d.	9/51 (17.6%)**
Hop growing farmers [139]	1/23 (4.3%)[b]	10/23 (43.5%)***	N.d.	3/23 (13.0%)*
Thyme growing farmers [140]	7/47 (14.9%)[b*]	16/46 (34.8%)***	N.d.	5/34 (14.7%)**
Chamomile growing farmers [140]	0/29 (0)[b]	4/29 (13.8%)	N.d.	3/29 (10.3%)*
Sage growing farmers [140]	1/32 (3.1%)[b]	11/32 (34.4%)***	N.d.	5/32 (15.6%)**
Herb industry workers [140]	4/40 (10.0%)[b]	14/40 (35.0%)***	N.d.	2/40 (5.0%)
Valerian growing farmers [141]	1/75 (1.3%)[b]	14/75 (18.7%)***	N.d.	3/15 (20.0%)**
Cattle and pig breeders [142]	2/50 (4.0%)[b]	20/50 (40.0%)***	N.d.	7/50 (14.0%)**
Rural respiratory patients [15, 30]	18/60 (30.0%)[a***]	154/312 (49.3%)***	135/222 (60.8%)***	N.d.
Rural and urban respiratory patients [16, 131	N.d.	180/720 (25.0%)***	81/340 (23.8%)***	N.d.
Healthy urban dwellers (reference group) [15, 30, 140]	7/100 (7.0%)[a] 1/50 (2.0%)[b]	8/170 (4.7%)[c]	1/49 (2.0%)	0/50 (0)

[a]Intradermal test;
[b]Prick test;
*-*** Significantly greater compared to reference group;
*P<0.05;
**P<0.01;
***P<0.001.
N.d = Not determined;
[c]All results of precipitin test were compared to this reference group which has been recognized as more reliable than the reference groups used later.

the *P. agglomerans* allergen is a very useful preventive test for the detection of workers who may develop hypersensitivity pneumonitis at continuing exposure.

Correlation of positive immunologic reactions to *P. agglomerans* in grain workers with reactions to other allergens associated with grain. The positive skin reactions to *P. agglomerans* in grain handlers were significantly correlated with those to grain dust extract (P<0.001), and grain weevil extract (P<0.05) [15, 30]. These results suggest that at least some of the positive reactions to grain dust extracts or grain weevil extracts observed in the skin testing of grain workers may, in fact, be due to reactions with *P. agglomerans* antigens present in these extracts.

Characteristics of the inhalation challenge method. The inhalation challenge with the use of a low dose of the *P. agglomerans* CA-S allergen, used by our group for the above-mentioned epidemiological examinations and for diagnostics of the hypersensitivity pneumonitis and asthma cases in which *P. agglomerans* was a one of suspected etiological agents (discussed in the next section), proved to be a reliable and safe test, both for scientific and diagnostic purposes. In the afore-mentioned tests, the CA-S allergen was strongly diluted in saline to the concentration of 20 µg/ml and was inhaled by each tested person in the total dose of circa 30 µg, equal to 0.3 µg of pure LPS, as determined by the *Limulus* test [118]. Such a dose was circa 100 times smaller compared to the doses mentioned by Rylander et al. [99] and Thorn [145] who assumed that the inhalation of 30–40 µg LPS seems to be a threshold level for inducing clinical symptoms and lung function changes in healthy human subjects. Thus, the decrements of spirometric values and subjective symptoms observed by us after the inhalation challenge with *P. agglomerans* extract, were not non-specific reactions caused by endotoxin, but specific reactions to *P. agglomerans* antigen(s), occurring only in sensitized individuals. No complications or worsening of the state of health of examined subjects were noted in the course of the nearly 1,000 tests performed.

Pantoea agglomerans as a specific cause of allergic pulmonary disorders (hypersensitivity pneumonitis, asthma) in humans

Hypersensitivity pneumonitis (HP). The first cases of hypersensitivity pneumonitis caused by *Pantoea agglomerans* were described Kuś in 1980 [16, 130]. This author described a group of 15 patients from the Lublin and Małopolska regions of eastern Poland who had a history of clinical manifestations after exposure to grain and/or flour dust, and diagnosis of HP. In all of them, *P. agglomerans* was identified as a one of the major causative agents of the disease, on the basis of inhalation challenge (which was not applied in 4 patients with a severe form of disease) and/or serological and skin tests. The patients' ages ranged from 40–69 years, 11 of them (73.3%) were women. The group consisted of 12 farmers who all reported the appearance of symptoms after exposure to grain dust at threshing (mostly when handling barley), one miller who reported symptoms after exposure to grain or flour dust, one baker who reported symptoms after exposure to flour dust, and one transportation worker who reported symptoms after exposure to grain or flour dust. With exception of one person, the majority of patients

did not related their ailments to work with mouldy plant materials, which could be partly explained by the fact that the Lublin region is characterized by a continental and not humid climate. The symptoms usually appeared 4–8 hours post-exposure and included dyspnea, chest tightness, cough, chest pain, sweats and fever. After a long-lasting exposure, the patients complained of malaise and general weakness. It is noteworthy that the decrements in spirometric values (VC, FEV_1) by 17–39% noted in the inhalation challenge with *P. agglomerans* CA-S (with low endotoxin content, causing no effect in the normal, non-sensitized subjects) also appeared after 4–8 hours, thus mimicking the natural exposure conditions. The decreases in spirometric values were accompanied by subjective symptoms (dyspnea, chest tightness, cough, fever, malaise).

In the group of HP patients described by Kuś [16, 130], there prevailed the chronic stage of disease which was diagnosed in 12 persons, whereas the acute and subacute stages of the disease were diagnosed in 1 and 2 persons, respectively. All 15 patients showed the presence of diffuse radiographic changes in the lungs, in the acute or subacute cases in the form of interstitial infiltrates of the ground-glass type (Fig. 14), whereas in the chronic cases in the form of micronodular and reticular changes, often with interstitial fibrosis and honeycombing (Fig. 16). In all, except for one patient, the patients' decrease of the lung function was noted, which was of restrictive pattern (in 8 persons) or mixed (restrictive + obstructive) pattern (in 6 persons). Patients with the chronic stage of the disease had fibrosis as well as other complications in the form of cor pulmonale (8 persons, 53.3% of the total group) and bronchiectases (1 person, 6.7% of the total). Scintigraphic examination revealed impairment of lung perfusion in most patients (Fig. 17). The patients were subjected to corticosteroid therapy together with the advice to avoid work at with exposure to grain dust, which succeeded in the remission of pathologic lung changes in the acute and subacute cases (Fig. 15), but not in chronic cases.

Five years later, Dutkiewicz et al. [146] described 2 cases of HP caused by *P. agglomerans* in female farmers from eastern Poland with a history of exposure to grain dust from wheat and barley. The etiologic role of this bacterium was proved by the results of inhalation challenge and the precipitin (double diffusion and immunoelectrophoresis) as well as skin tests.

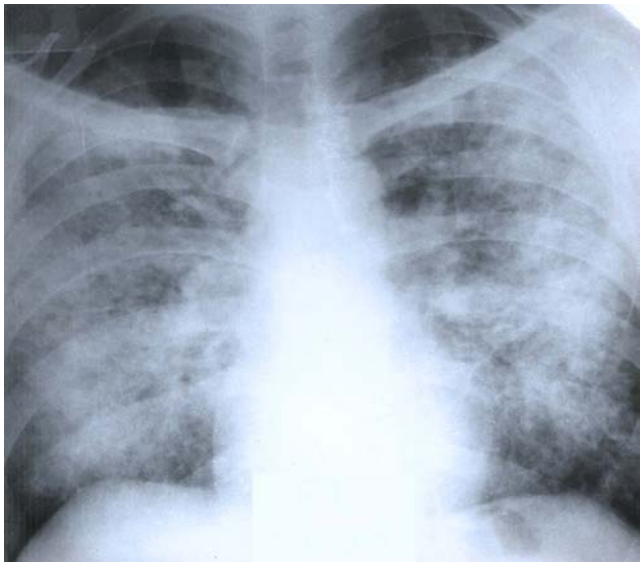

Figure 14. Chest radiogram of patient # 1, a male farmer with diagnosis of acute HP caused by *P. agglomerans*. Diffuse, confluent interstitial changes of the ground glass type are seen in all the lung fields. According to Kuś [131]

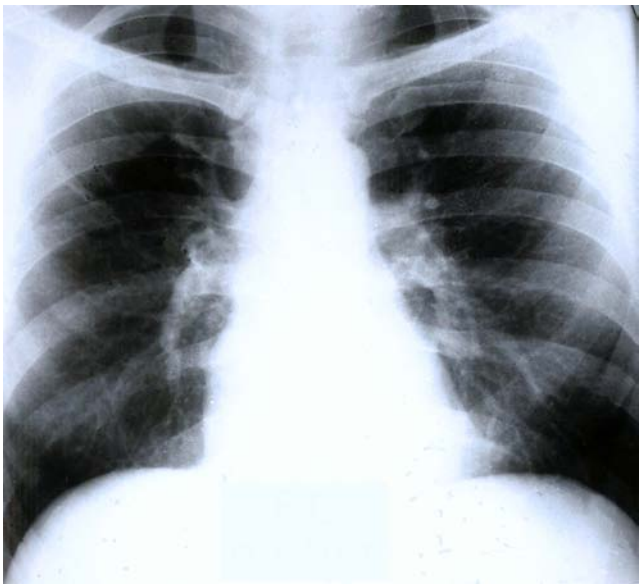

Figure 15. Chest radiogram of patient # 1 after 1.5-year corticosteroid therapy associated with avoidance of exposure to grain dust. A total remission of the changes is seen. According to Kuś [131]

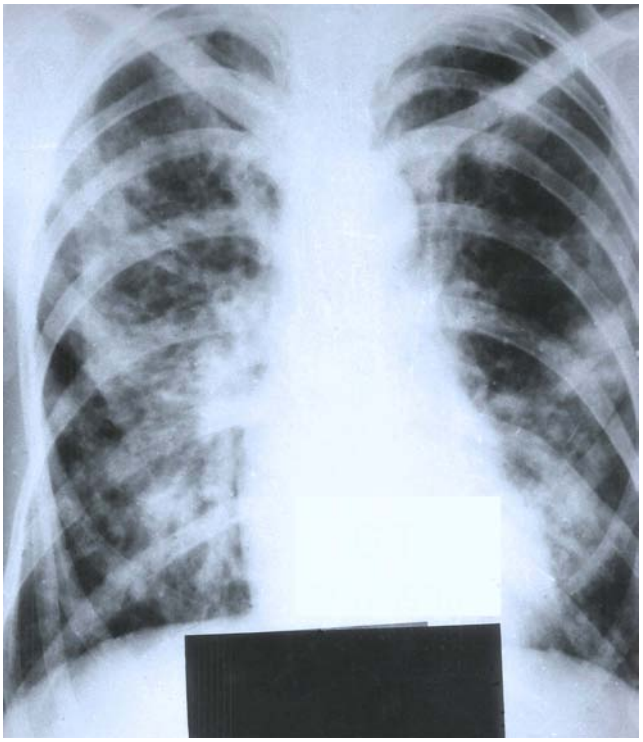

Figure 16. Chest radiogram of patient # 2, a female farmer with diagnosis of chronic HP caused by *P. agglomerans*. Interstitial fibrosis with honeycombing is seen in all lung fields. According to Kuś [131]

In 1988 and 1998, Milanowski [147] and Milanowski et al. [19] presented the complete characteristics of a group of 20 agricultural workers from eastern Poland with a history of clinical manifestations (mostly dry cough, shortness of breath, general malaise and chest pain) after exposure to grain dust, mostly barley (15 persons), dust from clover (4 persons), and dust from hay and grain (1 person); all

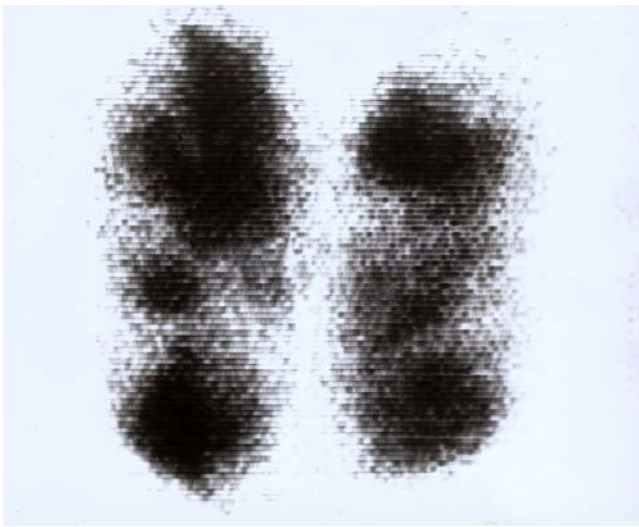

Figure 17. Scintigraphy of the lungs of patient # 3, a female farmer with diagnosis of chronic HP caused by *P. agglomerans*, with albumin labeled with ^{131}J. Diffuse multifocal deterioration of perfusion is seen in both lungs. According to Kuś [131]

had established diagnosis of HP. The group consisted of 14 males and 6 females aged 19–66 years, and comprised 10 farmers, 8 big grain elevator workers, 1 small grain store worker and 1 animal feed facility worker. The clinical tests were preceded by a detailed microbiological analysis of the samples of the offending plant material or dust causing disease symptoms. Patients were subjected to the inhalation challenge and immunological tests (agar-gel precipitation test, test for inhibition of leukocyte migration, intracutaneous test) with the extracts of the microorganisms dominant in particular samples, and with the extracts of *P. agglomerans* and *Saccharopolyspora rectivirgula*, the thermophilic actinomycete described as a common cause of HP [148].

On the basis of performed tests, *P. agglomerans* was identified as the cause of disease in 8 persons (40% of the total number of patients), in all cases as the dominant microorganism in the offending dust sample: in 4 farmers who developed symptoms after exposure to dust from clover, in 2 farmers who developed symptoms after exposure to dust from barley, in 1 farmer who developed symptoms after exposure to dust from mixed grain (barley + oats + corn), and in 1 worker of a small grain store, who developed symptoms after exposure to dust from mixed grain (barley + rye + oats). Four other species of mesophilic, non-branching bacteria were identified as causative agents of HP: *Arthrobacter globiformis* in 8 persons, *Alcaligenes faecalis* in 2 persons and *Brevibacterium linens* and *Staphylococcus epidermidis* in 1 each. Neither thermophilic actinomycetes nor fungi were identified as a cause of disease.

Of the 8 patients with HP caused by *P. agglomerans*, the acute stage of the disease was diagnosed in 4 farmers exposed to dust from clover, whereas the chronic stage was diagnosed in 3 farmers and 1 grain store worker exposed to grain dust. In chest X-ray, all the patients showed the presence of diffuse interstitial micronodular and reticular changes in lungs, mostly in the lower and middle lung fields; moreover, 2 of them had fibrosis. Pulmonary function tests showed restrictive type impairment in 1 patient, mixed type in 1 patient and normal spirometry in the remaining 6 patients. Bronchoalveolar lavage (BAL) examination showed a typical lymphocytic alveolitis, both in terms of percentage and absolute number of lymphocytes which was 20 times greater than in the healthy control subjects (P<0.001). The inhalation challenge with the diluted CA-S of *P. agglomerans* gave positive results in all patients, characterized by the decrement of VC and/or FEV_1 by at least 10% of initial value after 4–8 hours, associated with a significant increase in the number of leukocytes in peripheral blood, and often with general or respiratory symptoms (fever, muscle aches, malaise, headache, chest tightness); thus, similar to the group of patients described by Kuś [16, 130], mimicking the natural manifestations of the disease.

Mackiewicz et al. [149] described a case of HP in a female herb farmer heavily exposed to Gram-negative bacteria and endotoxin during the threshing of dried thyme (*Thymus vulgaris*). *Pantoea agglomerans*, which was present in the offending airborne dust, was identified as one of the main causative agents of the disease. The diagnosis was based on the presence of interstitial reticular changes in the lower parts of the lungs, restrictive impairment of lung function, the dominance of CD8+ over CD4+ in BAL fluid amounting to 2.8:1, and positive results of the inhalation challenge and the test for inhibition of leukocyte migration with the CA-S allergen of *P. agglomerans*. The results of the inhalation challenge, characterized by a significant decrease in spirometric values (VC, FEV_1, MEF 75) and occurrence of subjective symptoms (dyspnea, fever, general discomfort) mimicked in all details the symptoms noted after exposure to the offending thyme dust. To the best of our knowledge, this is the first case of HP due to inhalation of dust from herbs.

A case of HP in a farmer caused by *P. agglomerans* has been recently reported by Sennekamp et al. [150] from Germany. According to these authors, the HP cases caused by *P. agglomerans* and *Streptomyces albus* occur in Germany less frequently compared to those caused by thermophilic actinomycetes and fungi.

P. agglomerans, together with mesophilic actinomycetes, has also been implicated as a purported causative agent of respiratory disease resembling allergic alveolitis that occurred in autumn 1978 in a small Finnish community following repeated exposures to aerosol from tap water drawn from a lake, in a bath or in a sauna. In the agglutination test with flagellar antigen of *P. agglomerans*, significantly higher serum titres were found in the persons exposed to contaminated water and showing symptoms than in the unexposed control persons. However, the role of both *P. agglomerans* and mesophilic actinomycetes in the etiology of the disease could not be firmly established [151].

Bronchial asthma. *P. agglomerans* has been also considered as a potential causative factor of bronchial asthma. Durda et al. [152] demonstrated a significantly higher prevalence (P<0.01) of early positive reactions (after 20 min, identified by FEV_1 decrement by at least 10%) to the inhalation challenge with CA-S allergen of *P. agglomerans* in a group of patients with bronchial asthma (33.3%), compared to patients with chronic obturative bronchitis (12.0%). According to the authors, the inhalation challenge (performed as described in the previous section), is very useful both in the diagnostics and treatment of asthma, enabling a specific identification of allergen and selection of patients for a specific desensitization therapy. Skin tests with the CA-S allergen of *P. agglomerans*, performed by intracutaneous method, did not show a significant correlation with the inhalation challenge and, according to the authors, possess essentially a low diagnostic

value, especially after 8 and 24 hrs when a very high results of positive findings are recorded (85%-95%), suggesting non-specific reactions.

In the next study by Durda et al. [153], the superiority of the inhalation challenge with the *P. agglomerans* CA-S over intracutaneous test was confirmed. The frequencies of the early (after 20 min) positive inhalation reactions to the allergenic extracts of *P. agglomerans*, *Aspergillus fumigatus* and house dust, were similar (13.1%, 13.8%, and 10.4%, respectively), suggesting that *P. agglomerans* may play an important role in the etiopathogenesis of asthma, comparable to the two parallelly tested allergens, known as potent sensitizers. In two asthmatic patients with typical anamnesis indicating occurrence of symptoms after occupational exposure to grain dust, a clear-cut dual response to the inhalation challenge with *P. agglomerans* was noted, manifested by significant decreases in FEV_1 value after 2 minutes and then after 8 hrs [154]. Although the kind of asthma in the patients reacting to *P. agglomerans* has not been determined, rather a non-atopic form of this disease could be expected, as endotoxin present in the extract of this bacterium interferes with atopy [155]. The potential role of *P. agglomerans* in etiopathogenesis of non-atopic astma is probable, but needs verification by further research.

Promising results of immunotherapy with the *P. agglomerans* allergen. In 1980, Durda et al. [156] presented the results of specific desensitization therapy with increasing doses of CA-S allergen of *P. agglomerans*, which have been applied within one year to 36 asthmatic patients with an initial positive result of inhalation challenge with *P. agglomerans*, manifested by an at least 10% decrease in FEV_1. The results of the therapy were determined by two methods: measurement of FEV_1 value and assessment of the inhalation challenge with *P. agglomerans*, applied repeatedly to patients after one year-therapy. Both methods showed significant lung function improvement in the treated patients. FEV_1 increased significantly in 31 patients ($P<0.001$), on average by 611 ml. At the repeated examination by inhalation challenge after one year, only 2 out of 14 patients showed a positive response, indicating an improvement from 100% – 14.3%. The mean decreases in FEV_1 at 2 min post-challenge improved significantly from 479.3 ml to 46.4 ml, and at 20 min post-challenge from 362.9 ml to 53.6 ml ($P<0.01$). In 28 out of 36 treated patients (77.8%), there was improvement of the clinical state The problem which remains to be solved is whether endotoxin present in the extract could act as an adjuvant and influences the excellent results of the treatment.

Final conclusion. The results presented above demonstrate, on the example of *Pantoea agglomerans*, that non-branching bacteria occurring in organic dusts may cause specific allergic pulmonary diseases due to exposure to these dusts, such as HP and asthma. These results are supported by the reproduction of typical HP process in the experimental animals exposed to the inhalation of *P. agglomerans* allergen [20, 131], and the presence of the specific immune response to this allergen in the occupationally exposed populations [15].

This conclusion is important with relevance to the traditional but inaccurate view that only fungi or branching bacteria (actinomycetes), but not non-branching bacteria, may cause allergic diseases due to exposure to organic dusts. For example, in one review article on bioaerosol health effects [157], it is stated that 'most bacteria or bacterial agents are not very potent allergens, with the exception of the spore forming actinomycetes', but without any supporting citation. In fact, to the best of our knowledge, there are no convincing proofs that extrinsic allergic diseases could not be elicited by non-branching bacteria. On the contrary, the presence of endotoxin in Gram-negative bacteria may act, as mentioned earlier, as an 'auto-adjuvant' stimulating protein allergens to initiate a pathologic reaction.

Pantoea agglomerans as a specific cause of allergic skin diseases in humans

Śpiewak et al. [158] examined 75 students aged 16–23 years from agricultural schools in eastern Poland, with the test for inhibition of leukocyte migration in the presence of 3 environmental microbial allergens implicated in causing respiratory disease in farmers (*Pantoea agglomerans*, *Saccharopolyspora rectivirgula* and *Aspergillus fumigatus*), receiving 10 (13.3%) positive results with at least one allergen. The occurrence of positive cellular reactions to these allergens was significantly correlated with work-related symptoms and skin diseases. It is noteworthy that the diagnosis of allergic dermatitis was established in all three students who showed a positive cellular reaction to *P. agglomerans* (100%) and in only 7 out of the remaining 72 students (9.7%). This relationship was highly significant ($P<0.0001$) and firmly suggests the ability of *P. agglomerans* to evoke a skin disease by allergic reaction, independently of the toxic and irritative action of endotoxin produced by this bacterium.

In another study by Śpiewak et al. [159] on a group of 73 eastern-Polish farmers aged 16–84 years, growing hops and other crops, no significant relationship could be found between the cellular reactivity to *P. agglomerans* and occurrence of work-related skin symptoms. Nevertheless, the frequency of positive skin prick reactions to *P. agglomerans* was greater among farmers with work-related skin symptoms, compared to those without symptoms (2/14 (14.3%) vs. 1/59 (1.7%); $P<0.05$), which suggests that this bacterium might play a complementary role in the pathogenesis of work-related skin disorders associated with exposure to hops and hay [159]. In a 57-year-old female farmer with the established diagnosis of occupational allergic airborne and hand dermatitis to hops, the cutaneous late-phase reaction to *P. agglomerans* on prick test and the presence of specific precipitins to this bacterium in blood serum were noted, besides the strong reactions to hop extracts [160]. The authors ascribed a primary role in causing allergic disease to hop plant allergens, and a possible secondary role to *P. agglomerans*.

P. agglomerans has also been identified as one of the allergens (together with storage mites, flax allergens and moulds) that caused an occupation-related airborne contact dermatitis in a 41-year-old female farmer who had been exposed to plant dusts since childhood, and experienced skin inflammation symptoms mostly during threshing flax and milling dried herbs. The contribution of *P. agglomerans* allergen to the illness has been evidenced, similar to the aforementioned case, by the cutaneous late-phase reaction on prick test and the presence of specific precipitins in blood serum [161]. Here, it should be borne in mind that *P. agglomerans* occurs in the air at the levels of 10^4–10^5 CFU/m^3 during the processing of flax and some kinds of herbs (e.g. peppermint and chamomile), and at the level of 10^2 CFU/m^3 during hop processing, always forming above 40% of airborne Gram-

negative bacteria (Tab. 4). This might explain its possible role in causing allergic reactions associated with the handling of these crops.

Pantoea agglomerans as a specific cause of allergic diseases in farm animals

Pomorski et al. [18, 162] and Taszkun and Pomorski [163, 164] found a high incidence of allergic reactions to *Pantoea agglomerans* in cows tended on the territory of the Lublin Region of eastern Poland, and concluded that this bacterium is a potential causative agent of allergic alveolitis (hypersensitivity pneumonitis, granulomatous pneumonitis) and other pulmonary disorders in Polish cattle. In one study, carried out on 2 farms [18], cows with the symptoms of chronic pulmonary disease showed a significantly greater response rate to the allergens of *P. agglomerans* compared to healthy animals, both in the test for the presence of precipitins in blood serum (45.7% vs. 0%; P<0.001) and in the intradermal test (32.0% vs. 5.2%; P<0.01). It is noteworthy that in one animal an acute attack of dyspnea was observed after intradermal injection of *P. agglomerans* allergen. A total positive response of examined cows to *P. agglomerans* allergens was greater compared to other allergens (*Saccharopolyspora rectivirgula*, *Aspergillus fumigatus*), known as causative agents of allergic alveolitis in animals and humans. In another study, the presence of positive intradermal response to *P. agglomerans* on the territory of the Lublin region (including early, delayed and late reactions) was found in 56.8% of examined cows. The highest incidence of positive reactions (74.1%) was recorded in winter, while the lowest (33.3%) in summer. The total incidence of positive blood serum precipitin reactions was 25.1% and did not show a season-dependent variability [163, 164].

Only 5% of horses tended on the territory of the Lublin region showed the presence of anti-*P. agglomerans* precipitins in blood serum. No significant relationship was found between the presence of positive serologic reaction and occurrence of pulmonary symptoms, and the response rate to *P. agglomerans* allergen was smaller compared to other allergens implicated in allergic alveolitis (*Saccharopolyspora rectivirgula*, *Aspergillus fumigatus*). These results seem to indicate that the role of *P. agglomerans* in causing pulmonary disorders in horses is distinctly smaller compared to cattle [165].

Concluding remarks

The conclusion that *Pantoea agglomerans* is the most important hazardous agent present in grain dust and other agricultural dusts would be inaccurate. There is a multitude of other hazardous agents in these dusts, including: fungi that produce allergens, (1→3)-β-D-glucans, and mycotoxins, other bacterial species producing allergens, endotoxin, peptidoglycan and lipoteichoic acid (LTA), protein allergens produced by mites, insects, rodents and domestic animals, as well as various plant substances showing allergenic and/or toxic properties [4, 9, 43, 148, 166]. The development of individual agents depends on many factors associated with climate, soil and agricultural technology, and hence it is not possible to indicate one single hazardous agent which would be the most adverse worldwide.

Nevertheless, with all these reservations, *Pantoea agglomerans* should be regarded as one of the most important hazardous agents posing a risk of occupational disorders for agricultural workers, particularly for those exposed to the inhalation of grain dust. Such a statement is based on the bulk of evidence presented above in the current study concerning the common occurrence of this bacterium in grain dust, and its potent endotoxic and allergenic properties.

As mentioned in the Introduction, the role of *P. agglomerans* as an important health hazard associated with grain dust has been documented essentially by an interdisciplinary research group from one Polish city, and is generally underestimated in other countries of the world. There are various possible reasons for that, probably one is a lack of organizational and financial support similar to that provided by the US National Cotton Council for research on the effects of cotton dust, while the other is neglect of the problem by some researchers working on the effects of grain dust. While the role of *P. agglomerans* as a hazardous agent in grain dust was recognized by some prominent experts in this area, such as Lacey [9, 43] and Manfreda and Warren [4], some other scientists, even the authors of chapters in books on grain dust show surprising ignorance in this subject [167]. Regrettably, the problem is also neglected by the researchers who correctly recognize the risk posed by bacterial endotoxin present in grain dust, but overlook the need for identification of the source of such endotoxin [11, 95] which diminishes the value of their studies.

The research on endotoxin in cotton dust presented in the former article [23] serves as the best example of scientific and prevention benefits associated with the identification of bacterial species representing a main source of endotoxin in a particular dust. Identification of *P. agglomerans* as a source of endotoxin in cotton dust enabled the use and standardization of specific endotoxin produced by this species in the inhalation experiments in humans and animals, which made a significant contribution to the knowledge on ethiopathogenesis of byssinosis. Such identification also enabled the application of specific prevention measures reducing the growth of *P. agglomerans* on cotton plants and number of endotoxin-producing bacteria on raw cotton [166]. Without similar identification of the source of endotoxin in grain dust – whether *P. agglomerans* or other species – the experiments of low scientific value would be conducted with the use of *Escherichia coli* endotoxin, not associated with grain dust and much weaker compared to *P. agglomerans* [125], and a chance for the application of the specific prevention measures for reduction of bacteria and endotoxin on stored grain would be low. Also, lack of the identification of prevailing bacterial species prevents detection of the source of specific bacterial allergens in grain dust, which – as has been shown above on the example of *Pantoea agglomerans* – could be a significant cause of occupational allergic diseases, such as hypersensitivity pneumonitis, in spite of the outdated view on low allergenic potency of bacteria [157]. Obviously, identification of the appropriate allergenic species greatly enhances the diagnostics, treatment and prevention of the occupational diseases due to the inhalation exposure to organic dusts.

It is to be hoped that the future brings significant progress in the bacteriological studies of grain dust and other agricultural dusts, also in developing countries, to enable the identification of the source of endotoxin and allergens in grain dust, and application of the proper prophylactic measures which would allow for the reduction of cases of occupational disease among agricultural workers.

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