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Susceptibility of *Listeria monocytogenes* Strains Isolated from Dairy Products and Frozen Vegetables to Antibiotics Inhibiting Murein Synthesis and to Disinfectants

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Abstract

The susceptibility of 96 strains of *Listeria monocytogenes* isolated from food to antibiotics and disinfectants currently used in human therapy, veterinary, medicine and food industry was determined by a standard operating procedure – broth dilution method. Antimicrobial agents included the β-lactams ampicillin and penicillin, the lantibiotic nisin, and the disinfectants benzalkonium chloride and chlorhexidine gluconate. Among the studied strains we found 13 strains with 8-fold, 7 strains with 16-fold and 2 strains with 32-fold decreased susceptibility to ampicillin, as determined by MIC, compared to wild type reference strain. Interestingly, the mentioned strains were isolated from frozen vegetables and soups, none of the isolates from dairy products showed any elevated resistance to the studied antimicrobial agents. The occurrence in food products of strains with increased resistance to ampicillin is disquieting, especially since β-lactams are the most frequent antibiotic of choice in the therapy of infections caused by the pathogen.

Key words: *Listeria monocytogenes*, food-borne pathogens, antibiotic and disinfectant resistance, autolysis, autolysins

Introduction

Listeria monocytogenes (Hamon *et al*., 2006) is an opportunistic Gram-positive bacterium ubiquitous in nature: it occurs in waters, soil, rotting vegetation, animal faeces and wastewaters and is also isolated from faecal samples from healthy individuals – the bacterium has been found to be present in 5% of studied adults (Farber and Peterkin, 1991). *L. monocytogenes* is also found in such food products as fresh vegetables, milk, fish, poultry and meat. The spread of listeriae is related to the use of wastewaters for irrigation, which results in the presence of the bacteria on plants and products of animal origin. *L. monocytogenes* is an intracellular pathogen of humans and animals and is the causative agent of listerioses in the form of sporadic and epidemic infections (Portnoy *et al*., 2002; Hamon *et al*., 2006). Thirteen serotypes of *L. monocytogenes* have been identified and on average 90% of clinical infections are caused by serotypes Ia, Ib and IVb, the latter dominating in Europe (Schlech, 2000). However, a recent study embracing *L. monocytogenes* strains isolated from various sources in Poland has shown that the most prevalent is serotype IIa (Paciorek *et al*., 2006). The group at greatest risk for *L. monocytogenes* infection includes pregnant women, newborns and immunocompromised individuals, resulting in death in 25–30% of cases (Hamon *et al*., 2006).

The therapy of choice in the case of listeriosis involves a β-lactam, such as penicillin or ampicillin alone or in combination with an aminoglycoside, usually gentamicin (Charpentier and Courvalin, 1999). Alternative treatments, especially in the case of sensitivity to penicillin, involve trimethoprim in combination with a sulfonamide, such as sulfamethoxazole (Hof, 2004).

L. monocytogenes is naturally susceptible to penicillins, amino penicillins, carboxypenicillins, ureidopenicillins, carbapenems (*e.g.* imipenem) (Espaze and Reynaud, 1988; Troxler *et al*., 2000) but is relatively resistant to monobactams (aztreonam) and certain third generation cephalosporins, *e.g.* cefotaxim and ceftizoxim (Espaze and Reynaud, 1988). Poulsen *et al*. (1988) reported that 156 strains of *L. monocytogenes*

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from humans during 27 years (1958–1985) were susceptible to 12 antibiotics. The first case of a clinical strain of *L. monocytogenes* resistant to ampicillin was described in 1984 (Rapp *et al*., 1984; Pollock *et al*., 1986). More recent papers describe *L. monocytogenes* strains showing multiple resistance, mainly to aminoglycosides, chloramphenicol (Facinell *et al*., 1991; Tsakris *et al*., 1997), tetracycline and penicillin (Walsh *et al*., 2001; Poyart-Salmeron *et al*., 1992), erythromycin (Roberts *et al*., 1996), as well as trimethoprim (Charpentier *et al*., 1995), sulfamethoxazole and rifampin (Abrahim *et al*., 1998). The occurrence of resistant strains is usually related to the acquisition of transposons or plasmids carrying resistance determinants and transfer occurs mainly in the digestive tracts of animals and humans, frequently *Enterococcus* spp. and *Streptococcus* spp. (Charpentier and Courvalin, 1999; Charpentier *et al*., 1995). The transfer of vancomycin resistance from enterococci to *L. monocytogenes* by conjugation under laboratory conditions has also been shown (Biavasco *et al*., 1996). Recently 2 cases of listeriosis caused by penicillin-resistant *L. monocytogenes* and 6 by strains resistant to ampicillin were described but these strains, like the ampicillin-resistant one mentioned above, were never characterized (Safdar and Armstrong, 2003). The resistance of some *L. monocytogenes* isolates to popularly used disinfectants, especially chlorine compounds such as chlorhexidine and benzalkonium chloride, has been described. The mechanism of adaptation or resistance to benzalkonium chloride is associated with efflux pumps (Soumet *et al*., 2005). Two efflux pumps have been described in *L. monocytogenes*: MdrL can extrude antibiotics (macrolides and cefotaxime), heavy metals and ethidium bromide (Mata *et al*., 2000) and Lde is associated with fluoroquinolone resistance and partly responsible for resistance to acridine orange and ethidium bromide (Godreuil *et al*., 2003). A recent study has shown that MdrL is at least partly responsible for adaptation to benzalkonium chloride (Romanova *et al*., 2006).

The aim of the present study was to determine the susceptibility to ampicillin, penicillin, nisin, benzalkonium chloride and chlorhexidine of 96 food isolates of *L. monocytogenes*. All these agents are or have been used in medicine, veterinary medicine and the food industry. We have also attempted to elucidate the reason for the reduced susceptibility of several of the studied strains to ampicillin.

Experimental

Material and Methods

Bacterial strains. We studied 96 strains of *L. monocytogenes* isolated from frozen food (vegetables and soups) collected by Sanepid in Bydgoszcz (WSSE, Bydgoszcz). All strains were classified as *L. monocytogenes* according to the Polish norm PN-EN ISO 11290-1:1999 and further characterized in our Department by streaks on Palcam and OCLA agar plates (Oxoid). We also did a haemolysis test on TSYEB agar plates (Tryptone Soy Agar slants with 3% yeast extract; BioMérieux; BTL) containing 5% (v/v) sheep blood. The reference strain used in these studies was *L. monocytogenes* EGD. Bacteria were grown in TSYEB at 37°C with mild shaking (120 rpm) to mid-exponential phase of growth. Strains were stored at 4°C on TSYEB agar plates.

Antimicrobial agents. The antibiotics tested were: ampicillin, penicillin, nisin (Sigma) and the disinfectants: benzalkonium chloride (alkyl dimethyl benzyl ammonium chloride) and chlorhexidine gluconate (Sigma). Antimicrobial stock solutions were prepared in distilled water and stored at –20°C.

Broth dilution method for minimal inhibitory concentration (MIC) determinations. MICs of antimicrobial agents for food isolates were determined by broth microdilution method of the National Committee for Clinical Laboratory Standards (NCCLS, 2003), currently the Clinical and Laboratory Standards Institute (CSLI). The final concentrations of the antibiotics were 0.0468 – 12 μg/ml and for nisin 1 – 40 μ/ml. Each MIC determination was repeated two times.

Isolation of cell wall. *L. monocytogenes* cells were harvested by centrifugation (6 000 × g, 15 min at 4°C) and resuspended in 1/40 of the original culture volume of ice-cold saline. Glass beads (diameter 150–215 mm; Sigma) were added (1 g per 1 ml cell suspension) and ten 1 minute bursts of ultrasound waves were employed in VCX-600 ultrasonicator (Sonics and Materials, USA) at amplitude 20%. The crude cell wall preparation was sedimented by centrifugation in a Beckman centrifuge (25 min, 100 000 × g at 4°C). The cells were washed in appropriate buffer and then resuspended in it.

Autolysis of crude cell wall preparations from *L. monocytogenes*. After sonication as above, the cell walls were sedimented by centrifugation, washed in 10 or 50 mM Tris-HCl buffer and resuspended in the same buffer pre-warmed to 37ºC. The suspension was incubated with shaking at 37ºC and changes in absorbance were followed at 600 nm.

Autolysis of cells. Cultures of *L. monocytogenes* strains were grown to early exponential phase (OD_{600} 0.20) in TSB broth. To determine the effect of the antibiotic on growth, 10 times the MIC of penicillin for each of the individual strains, or 0.5 times the MIC of nisin was added, and the response of the culture was followed spectrophotometrically (Novaspec II spectrophotometer LKB-13 Pharmacia) while continuing incubation at 37°C with shaking. To determine the effect of Triton X-100 or SDS, the cells were grown to OD_{600} ~ 0.6, harvested and resuspended in 50 mM Tris-HCl (pH 7.5), 0.1% (v/v) Triton X-100 or 1% (v/v) SDS. Lysis of the cell suspension at 37°C was followed spectrophotometrically at 600 nm.

Induced cell lysis. Cultures of *L. monocytogenes* strains were grown to mid-exponential phase (OD_{600} 0.80) in TSB broth. Cells were harvested by centrifugation as above and resuspended in 50 mM Tris-HCl (pH 7.5) buffer pre-warmed to 37°C. To determine the effect of muramidase-induced lysis lysosyme or cellosyl was added to final concentration 2 mg/ml, and the lysis of the culture was followed spectrophotometrically while continuing incubation at 37°C with shaking.

Extraction of surface-associated autolytic enzymes. Cells of *L. monocytogenes* were harvested by centrifugation, washed in saline and resuspended in an ice-cold solution containing 4 M LiCl and 0.5 mM phenylmethylsulfonylfluoride (PMSF) in 50 mM Tris-HCl buffer, pH 7.0. The suspension was stirred for 30 min with the use of a magnetic bar in an ice bath, after which the cells were harvested (20 000 × g, 10 min at 4°C). The supernatant was transferred to dialysis tubing with cutoff value 3 500 (Spectrum) and dialyzed for 12 hours at 4°C against 50 mM Tris-HCl, pH 7.0 containing 100 mM LiCl, with several changes of the buffer. Alternatively, cells of *L. monocytogenes* were extracted with 1% (v/v) SDS at room temperature for 5 min, after which the cells were harvested (20 000 × g, 10 min at 4°C). The supernatant was analyzed on 12% SDS-polyacrylamide gel and renaturing gel.

Haemolytic activities. Bacteria were cultured on sheep blood agar at 37°C for 36 h. *L. innocua* and *L. ivanovii* were used as a negative and positive control, respectively. After incubation, the narrow ring of β-hemolysis produced by the strains was compared with that of *L. monocytogenes* EGD.

Plasmid DNA isolation. Plasmid DNA from *E. coli* was isolated and purified with the Plasmid Miniprep Plus kit (A&A Biotechnology). The procedures for the isolation of plasmid and chromosomal DNA from *L. monocytogenes* were performed as previously described (McLaughlan and Foster, 1998), starting with digestion of the bacterial cell wall in 5–10 mg/ml lysozyme-containing GTE buffer for 1 h at 37°C.

Penicillin binding protein (PBP) assay. The PBP pattern of *L. monocytogenes* was visualized by fluorography following electrophoresis of cell membrane proteins incubated with [H^3]-benzylpenicillin, as described in detail elsewhere (Korsak *et al*., 2002).

Results

Antimicrobial resistance. We analysed 96 strains of *L. monocytogenes* isolated from frozen meal and dairy products study for susceptibility to antimicrobial agents. Eighteen of the strains were just as sensitive to ampicillin as the reference strain (EGD) used (MIC 0.156 μg/ml), 14 were more sensitive to the antibiotic (0.039–0.078 μg/ml) and 65 of the strains showed reduced susceptibility to ampicillin (0.3125–5.0 μg/ml). Two of the latter strains were resistant to 5 μg ampicillin/ml, which is 32 times the MIC for EGD. None of the isolated strains showed any significantly altered susceptibility to nisin, the MIC values for the antibiotic against the isolates being similar as for EGD.

Twelve of the studied strains were as susceptible to chlorhexidine as EGD (MIC 1.25 μg/ml) whereas all the remaining strains had two to 4-fold reduced susceptibility to the disinfectant (MIC 2.5–5 μg/ml). In the case of benzalkonium chloride, the sensitivity of 37 of the strains to the compound was the same as that of EGD (MIC 1.25 μg/ml). Only 7 of the strains were two to 4-fold less sensitive (MIC 2.5–5 μg/ml) (Table I).

For further, more detailed analysis, six of the tested strains (Table II) were selected. Worth note is that strain was 11 times less sensitive to penicillin than EGD, 16 times less susceptible to ampicillin and 2 times less susceptible to both studied disinfectants. Similar values were obtained for strain 21 which, however, it was not more resistant to penicillin. Literature data indicate that one of the main reasons for the resistance of *L. monocytogenes* to antibiotics is the presence of mobile genetic elements carrying resistance genes. Multiple resistance of *L. monocytogenes* has been shown to be related to the presence of specific genes carried by plasmids pIP811, pUBX1 and pWDB100, whereas resistance to trimethoprim is determined by dihydrofolate reductase gene (*dfrD*), present in the 3.7 kb broad host range plasmid pIP823 (Charpentier and Courvalin, 1999).

In this study we attempted to identify putative genetic elements that could carry genes responsible for the observed elevated resistance to ampicillin. *L. monocytogenes* has recently been shown to carry a chromosomal

Table I
Susceptibility of *L. monocytogenes* isolates to ampicillin and disinfectants

MIC (μg/ml)	Number of strains	MIC (μg/ml)	Number of strains
Ampicillin		Chlorhexidine	
0.039	11	1.25	12
0.078	3	2.5	43
0.156	18	5.0	7
0.3125	18	Benzalkonium chloride	
0.625	25	1.25	37
1.25	13	2.5	6
2.5	7	5.0	1
5.0	2		

Table II
Antimicrobial susceptibility of selected strains of *L. monocytogenes*

Strain designation	Origin	MIC (μg/ml)				
		ampicillin	penicillin G	chlorhexidine	benzalkonium chloride	nisin
1	cauliflower	2.5	0.7	5.0	2.5	9
6	vegetables with rice	5.0	0.12	2.5	2.5	10
21	vegetables soup	2.5	0.06	5.0	5.0	11
58	green peas	5.0	0.12	2.5	2.5	10
105	dairy product	0.156	0.06	2.5	1.25	10
121	dairy product	0.156	0.03	2.5	1.25	11
EGD	collection of Institute of Microbiology	0.156	0.06	2.5	1.25	11

gene *Lmo0540* that may code a putative class C β-lactamase (Guinane *et al*., 2006) but we have never been able to demonstrate any-lactamase activity in this bacterium using a number of different approaches (Poroś-Głuchowska, 2004). Similarly, to our knowledge no chromosomally coded *L. monocytogenes* β-lactamase activity has been described by any other laboratory. Subsequently, we used a specific procedure for the isolation of plasmid DNA from *L. monocytogenes* strains, involving a phenol step, followed by electrophoresis in 1% agarose gel. In spite of repeated attempts in no case any plasmid preparations were obtained from the selected *L. monocytogenes* strains.

In the next stage we analysed the PBP pattern of the studied strains in order to see if any quantitative or qualitative differences explaining the increased resistance to ampicillin could be observed. No differences were observed (data not presented). In turn, we decided to carry out a physiological analysis aimed to demonstrate changes in the protein composition of the cell wall and/or muropeptide composition of the cell

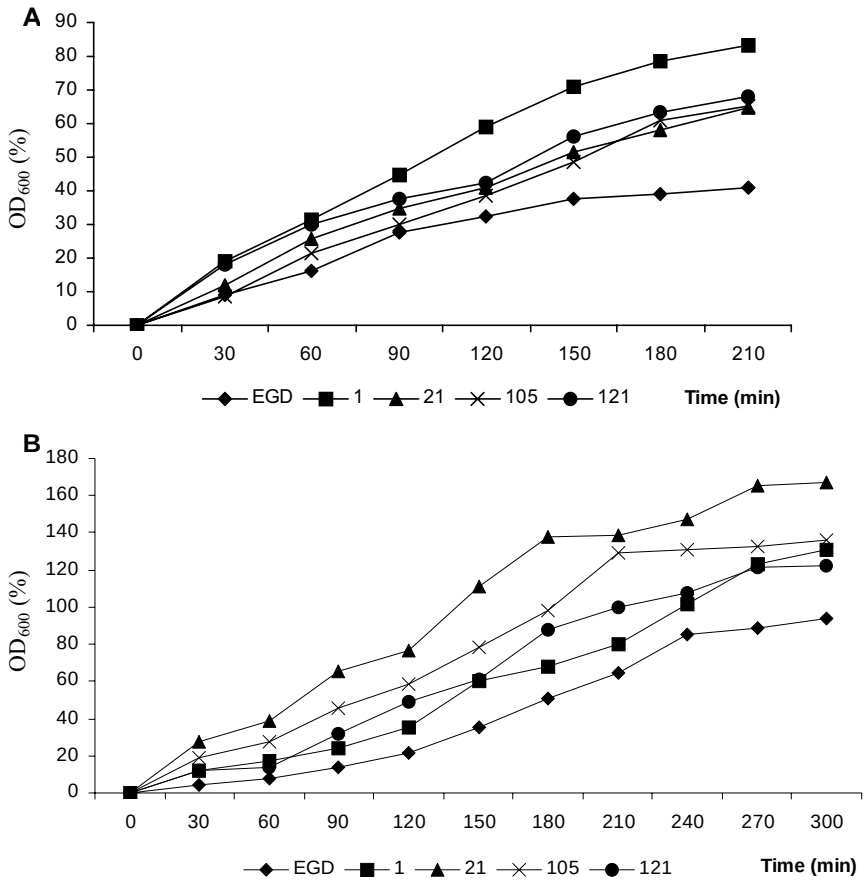

Fig. 1. Effect of antibiotics at 10 times the MIC on the growth of *L. monocytogenes* EGD and selected isolates
A) penicillin; B) nisin

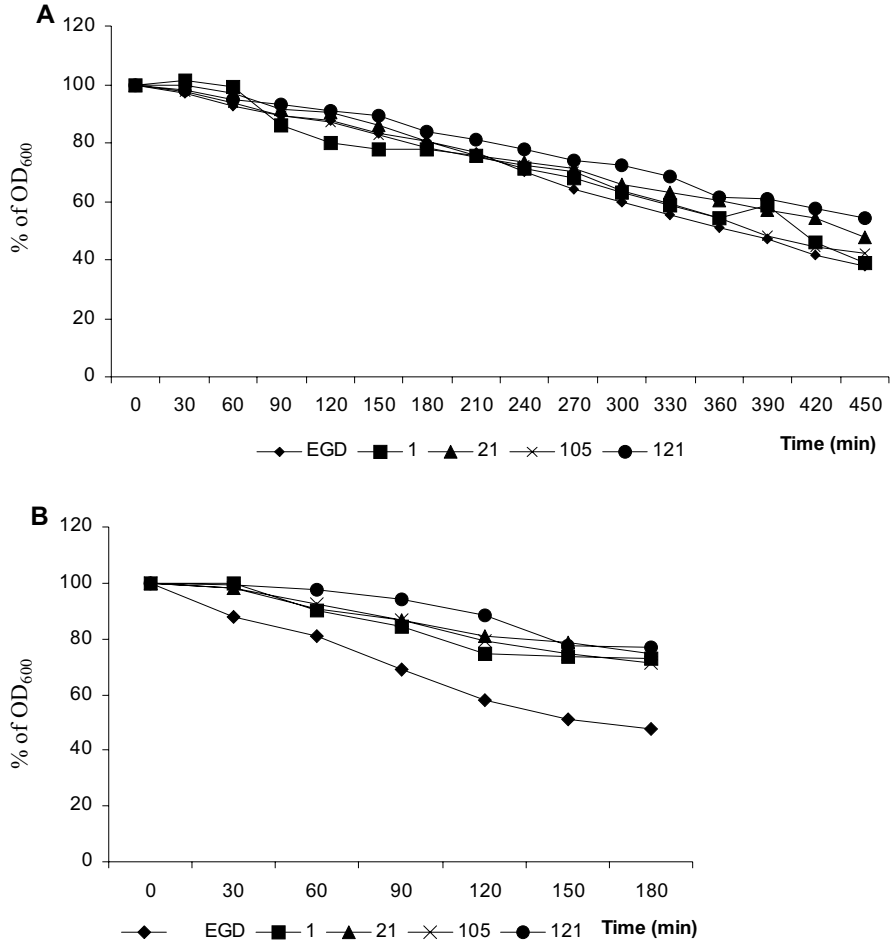

Fig. 2. Autolysis of cells of *L. monocytogenes* EGD and selected strains induced by detergents
A) Triton X-100; B) SDS

wall murein, as possible determinants for the observed resistance to β-lactams, *i.e*. impeding the penetration of the antibiotic to the cytoplasmic membrane.

Autolysis of whole cells and isolated cell walls. A significant effect of penicillin G on the growth of the studied strains was observed – all the isolates were less susceptible compared to EGD at ten times the MIC for the individual strains, and this was very evident already after 60 min of the experiment. Strain 1 grew faster by about 37% and the remaining strains by about 23% (Fig. 1A). Similarly, all the studied isolates demonstrated strongly reduced sensitivity to nisin at 0.5 times the MIC compared to strain EGD. The growth rate of strain 21, for which the MIC of nisin was equal to that of the reference strain, was faster by about 74% than EGD (Fig. 1B). All the strains were also slightly less susceptible to the action of Triton X-100, compared to strain EGD, but the rate of their autolysis was similar. The least susceptible to the action of the detergent were strains 21 and 121, which autolysed slower by 10% and 17% after 450 min, respectively, compared to EGD (Fig. 2A). When SDS was used all the studied strains were less susceptible to the action of the detergent than the wild-type strain. The autolysis of strain 121 was about 30% slower, and that of the remaining strains by about 25% slower than observed for EGD (Fig. 2B). Murein isolated from the strains 1, 21 and 121 showed faster autolysis kinetics than for that from strain EGD – in the case of strain 1 by as much as 24%. A similar tendency was observed for murein from strains 21 and 121, which lysed 8% faster than murein from strain EGD. Only murein from strain 105 showed a slightly slower rate of autolysis (by about 4%) (Fig. 3).

Cell lysis. The cells of all the studied strains lysed much faster in the presence of hen egg white lysozyme than EGD. The rate was faster by 12% for strain 121 and by 7% for strain 1 (Fig. 4A). Lysis of whole cells by cellosyl was somewhat faster (by 4 to 16%) for three of the strains, but strain 21 was lysed slower by about 6 % compared to the wild type EGD (Fig. 4B).

Analysis of surface protein composition. Analysis of the protein fraction isolated with the use of SDS suggests similar protein composition of strain 1 and the reference strain EGD, as well as between the pairs of strains 58 and 105, and 6 and 21 (Fig. 5). The profiles of the reference strain and strain 1 lack a protein

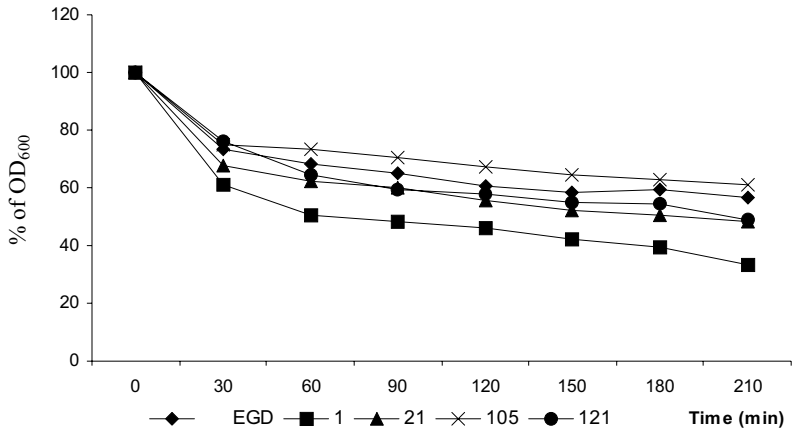

Fig. 3. Autolysis of crude murein preparation isolated from EGD and selected *L. monocytogenes* strains

with mass about 60 kDa (designation c, Fig. 5) and there is also much less of a protein (or two proteins with similar mass) located between 20.1 kDa and 14.4 kDa (designation e, Fig. 5). The surface protein fraction of strains 58 and 105 contains a clearly visible protein with mass approx. 94 kDa (designation a, Fig. 5), this protein being produced by the remaining strains in much smaller copy number. Strains 6 and 21 are characterized by a decidedly different profile, which lacks a protein between 94 kDa, a 67 kDa (designation b, Fig. 5) present in the remaining strains as well as two proteins between 67 kDa and 43 kDa, that are visible in strains 1, 58 and 105 (designation d, Fig. 5).

Analysis of the surface fraction isolated using LiCl shows multi-point similarity in the protein composition for the reference strain and strain 1, as opposed to strains 6, 21, 58 and 105 (Fig. 6). Three proteins that were

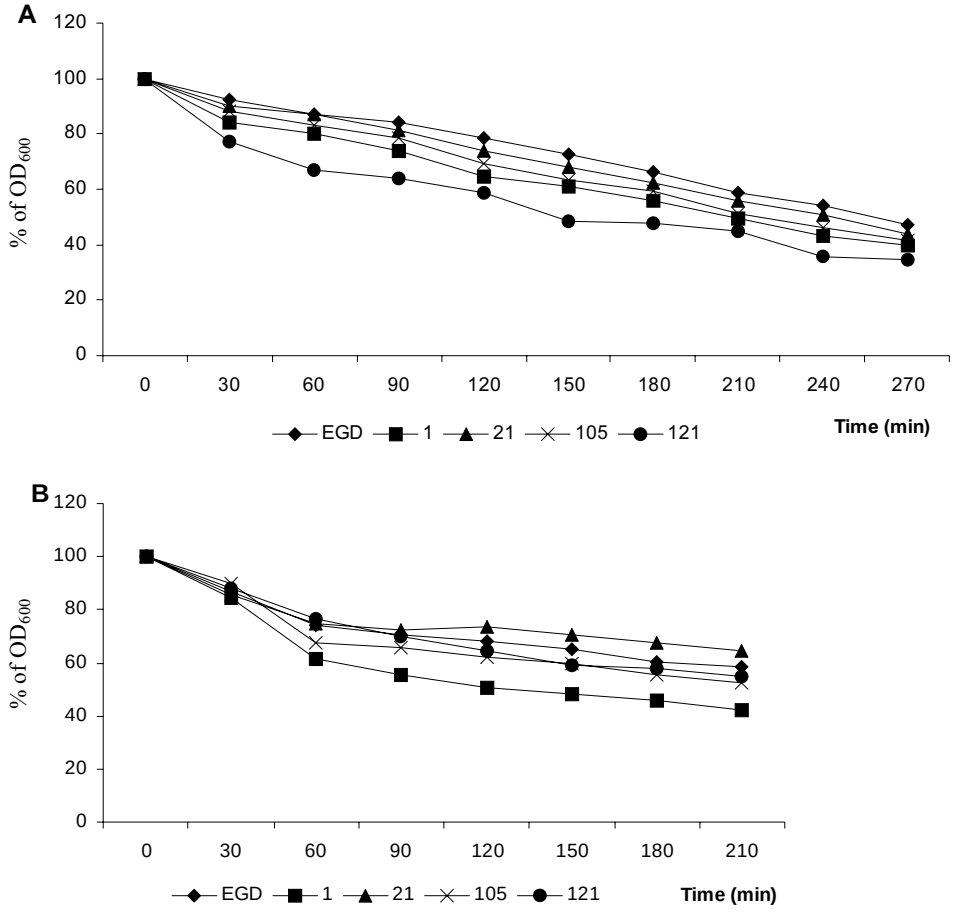

Fig. 4. Kinetics of lysis of *L. monocytogenes* EGD and selected strains by murein-degrading enzymes A) lysozyme; B) cellosyl

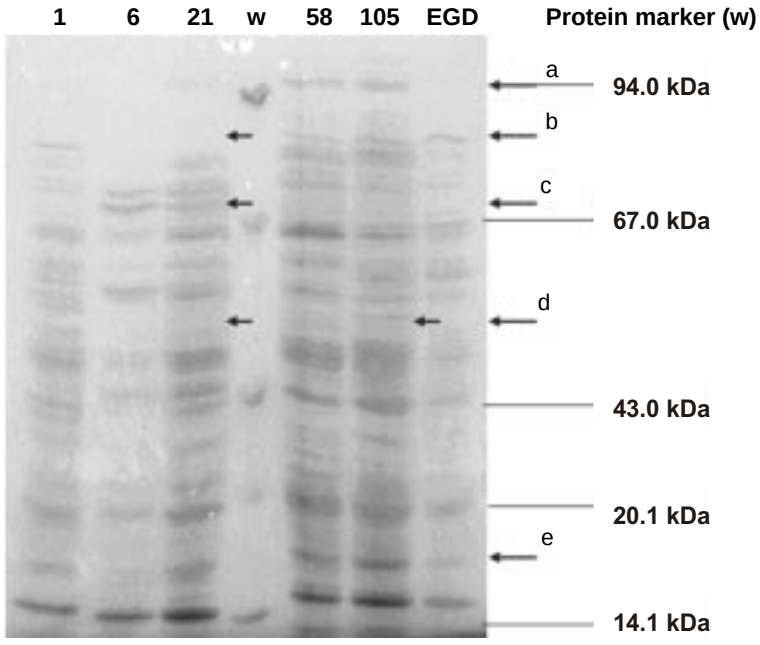

Fig. 5. Electrophoregram of surface proteins of selected *L. monocytogenes* strains and reference strain EGD isolated using SDS. The numbers above the lanes indicate the individual strains tested, lane w – molecular weight markers**.**
The letters a-e indicate differences in protein pattern. Explanations in the text.

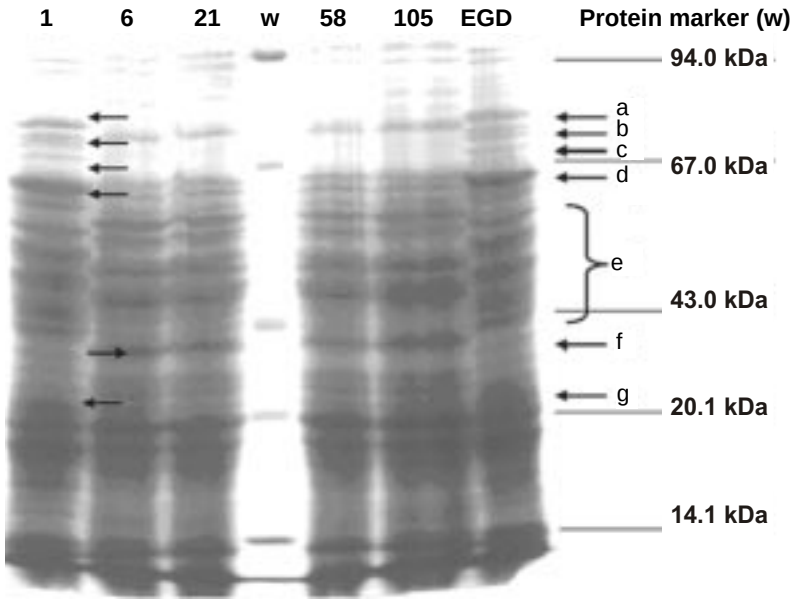

Fig. 6. Electrophoregram of surface proteins of selected *L. monocytogenes* strains and reference strain EGD isolated using LiCl. The numbers above the lanes indicate the individual strains tested, lane w – molecular weight markers**.**
The letters a-g indicate differences in protein pattern. Explanations in the text.

present only in the reference strain and strain 1, located between 94 kDa, and 67 kDa were observed (designation a, b, c, Fig. 6) as well as two additional ones in the protein profile of the above strains – approx. 65 kDa (designation d, Fig. 6) and approx. 23 kDa (designation g, Fig. 6). Significant quantitative and/or qualitative differences were also observed among proteins in the *ca*. 60 kDa to *ca*. 40 kDa range (designation e, Fig. 6). An additional protein with mass *ca*. 38 kDa, or its increased amount compared to the reference strain, that was not present in strain 1, was observed in the case of strains 6, 21, 58 and 105 (designation f, Fig. 6).

Discussion

An undesirable consequence of the use, frequently unwarranted, of antimicrobials is the occurrence and spread of strains of pathogenic bacteria resistant to antibiotics and/or disinfectants in the environment. This problem is particularly challenging in the case of food-borne pathogens such as *L. monocytogenes*, which accounts for 28% of food-related deaths in the USA (Mead *et al*., 1999). The incidence of listeriosis has increased over the past two decades throughout the world. Several large food-borne outbreaks of listeriosis

have been reported in numerous countries, including England, Germany, Sweden, New Zealand, Switzerland, Australia, France, and the US. *L. monocytogenes* strains resistant to antibiotics were reported in the eighties (*e.g.* Rapp *et al*., 1984; Poulsen *et al*., 1988). Since then, *Listeria* spp. isolated from food, the environment or in sporadic cases of human listeriosis have been shown to be resistant to one or several antibiotics and the number of reports of isolates of *L. monocytogenes* resistant to β-lactam antibiotics, the antibiotics of choice for the treatment of listeriosis, is slowly but systematically growing (Rapp *et al*., 1984; Walsh *et al*., 2001; Chen *et al*., 2001; Prazak *et al*., 2002; Safdar and Armstrong, 2003). The data published by Prazak *et al*. (2002) indicate that 87% of multiply resistant strains were resistant to penicillin and some of these in addition, also to gentamicin, the second antibiotic used together with a β-lactam in combined chemotherapy of listeriosis. An equally disturbing phenomenon is the adaptation or resistance of *L. monocytogenes* to benzalkonium chloride and other disinfectants (Romanova *et al*., 2006).

The data for Poland are scarce, this being due, among others, to infrequent screening for the occurrence of *L. monocytogenes*. A fairly recent study involving 73 strains of *L. monocytogenes* isolated from a variety of clinical and environmental sources screened for resistance to ampicillin, penicillin, gentamicin, erythromycin, clarithromycin, sulfisoxazole and trimethoprim found that the strains were susceptible to the antibiotics studied, except for the resistance of 30.1% of the strains to sulfisoxazole (Paciorek, 2004). In our earlier studies, we showed that *L. monocytogenes* can readily develop resistance to β-lactams under antibiotic pressure as well as under transposon mutagenesis conditions (Poroś-Głuchowska and Markiewicz, 2003a; 2003b). The pathogen is capable of acquiring antibiotic resistance genes from enterococci and streptococci through movable genetic elements such as transposons and plasmids and resistance genes also come from other Gram-positive and Gram-negative bacteria through conjugative mobilization (de Niederhausern *et al*., 2004; Bertrand *et al*., 2005).

In this study, a collection of 96 *L. monocytogenes* strains isolated from dairy products and frozen foods was screened for susceptibility to commonly used antibiotics and biocides. Analysis of the growth rate of the strains isolated from food products at 37°C in TSB did not indicate any significant differences compared to EGD, in spite of their being subjected to thermal processing (pasteurization, freezing). This seems to indicate that temperature variations were not a factor contributing towards observed resistance (Asselt and Zwietering 2006).

Of the strains studied, 68% showed reduced susceptibility to ampicillin, and for 2 of these the MIC of ampicillin was approx. 32-fold higher than for strain EGD. Interestingly, the resistance of these strains to penicillin was in a much lower range, from 2 to 12-fold the MIC for EGD. In our *in vitro* attempts to generate ampicillin resistant derivatives of *L. monocytogenes* EGD mentioned above (Poroś-Głuchowska and Markiewicz, 2003a; 2003b), the highest value was similar to that observed for two of the studied isolates, that is 32-fold. Attempts to identify the cause of ampicillin resistance of the food-borne strains were negative and, as mentioned in the introduction, no resistance mechanism was identified for any of the other β-lactam resistant isolates mentioned in the literature. An interesting observation was the behaviour of the isolates showing lower susceptibility to penicillin to the presence of the antibiotic at 10 times the individual MICs. Penicillin is well known to kill *L. monocytogenes* after a lag phase of several hours, without accompanying lysis (Chen *et al*., 1996; Popowska *et al*., 1999). This is reflected as a plateau in the growth curve after the addition of penicillin at concentrations above the MIC. The isolates, however, continued to grow, as is clearly visible in Fig. 1A. Nisin does not affect the growth of *L. monocytogenes* the way penicillin does, but nevertheless in this case the isolates grew much faster at 0.5 times the MIC for the individual strains than the reference strain during the time of the experiment. These observations demand further investigation.

In the case of benzalkonium chloride 16% of the strains were characterized by from 2 to 4-fold reduced susceptibility, whereas as many as 84% of the isolates were equally susceptible to the compound as EGD. The ratio was practically quite the opposite for chlorhexidine in which case 82% of the studied strains showed 2 to 4-fold reduced susceptibility to the disinfectant whereas 18% were just as susceptible to the biocide as EGD. It is assumed that reduced susceptibility to disinfectants can be a result of altered permeability of the bacterial cell wall, *e.g.* structural modifications, thickness, or a consequence of the action of active pumps (*e.g.* MdrL), which detoxify the cell (Mata *et al*., 2000; Romanova *et al*., 2006). Since we ruled out β-lactamase activity and changes in the PBPs of *L. monocytogenes* as the reasons for the resistance of some of the isolated strains to ampicillin and penicillin, and it is known that changes in cell wall permeability may affect the resistance of some bacteria to antibiotics (*e.g.* Sieradzki and Markiewicz, 2004), we decided to examine the cell wall murein of the mutants.

A comparison of the muropeptide profiles of the mutants obtained after HPLC chromatography of a muramidase digest of murein did not reveal any significant changes in the murein of the strains showing

elevated resistance to β-lactams. There were some minor changes in the murein of the strains with reduced susceptibility to the disinfectants, but in view of only two-fold reduction in the susceptibility of the strains to the compounds, these observations were not followed up. Differences were, however, observed when crude murein preparations from the cells were subjected to conditions promoting autolysis. Murein from strain 105 reproducibly autolysed slower than that from EGD, whereas murein from strains 1, 21 and 121 autolysed much faster than murein from the reference strain. These results may point to differences in the modification of murein, such as extent of *N*-acetylation of aminosugars or the presence or absence of *O*-acetyl groups which may affect the activity of the autolytic enzymes in the cell wall (Popowska, 2004). Alternatively, the studied strains may lack certain autolytic enzymes or the enzymes in question may be less or more stringently regulated. These conclusions are supported by the differences in the kinetics of lysis of intact *L. monocytogenes* cells induced by Triton X-100 and SDS. Similar differences were also observed when *L. monocytogenes* cells were treated with egg white lysozyme or the muramidase cellosyl. Both enzymes not only hydrolyze bonds in murein but also induce the activity of autolysins, especially lysozyme, which is a protein with cationic nature (Leitch and Willcox, 1999). Altogether, in the light of these observations further experiments are being planned.

The greatest differences between the strains were found when the surface protein profiles of the cells were compared, regardless of extraction method used. Compared to EGD, the studied isolates either had new bands not observed in the reference strains or lacked certain proteins. These differences may reflect, for instance, the absence of certain autolytic activities or the presence of proteins whose synthesis is induced under temperature stress conditions and which may determine altered permeability of the cell wall.

Much remains to be elucidated, but the main conclusion of these studies is that strains of *L. monocytogenes* resistant to β-lactam antibiotics are becoming increasingly more ubiquitous in the environment, especially in food products. The increasing incidence of antibiotic resistance in *L. monocytogenes*, in combination with the widespread use of antibiotics in medicine and various branches of industry may have significant future clinical implications for the treatment of listeriosis.

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