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Biodiversity of *Lactococcus lactis* bacteriophages in Polish dairy environment☆

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We present here the results of an exploration of the bacteriophage content of dairy wheys collected from milk plants localized in various regions of Poland. Thirty-three whey samples from 17 regions were analyzed and found to contain phages active against *L. lactis* strains. High phage titer in all whey samples suggested phage-induced lysis to be the main cause of fermentation failures. In total, over 220 isolated phages were examined for their restriction patterns, genome sizes, genetic groups of DNA homology, and host ranges. Based on DNA digestions the identified phages were classified into 34 distinct DNA restriction groups. Phage genome sizes were estimated at 14–35 kb. Multiplex PCR analysis established that the studied phages belong to two out of the three main lactococcal phage types — c2 and 936, while P335-type phages were not detected. Yet, analyses of bacterial starter strains revealed that the majority of them are lysogenic and carry prophages of P335-type in their chromosome. Phage geographical distribution and host range are additionally discussed.

Keywords: bacteriophages, *Lactococcus lactis*, biodiversity, dairy environment

INTRODUCTION

Bacteriophage attack is regarded as a serious problem in the dairy industry worldwide. Over the years, this circumstance has initiated extensive studies on bacteriophages occurring in dairies in Europe and other continents (Jarvis, 1977; Lembke *et al.*, 1980; Relano *et al.*, 1987; Powell *et al.*, 1989; Prevots *et al.*, 1990; Moineau *et al.*, 1992; 1996; Casey *et al.*, 1993; Miklic & Rogelj, 2003; Sanlibaba & Akcelik, 2005). Regardless of sanitary precautions, starter strain rotation or constant development of new phage-resistant bacterial strains, bacteriophage infections persist in the dairy industry (Sing & Klaenhammer, 1993; Moineau *et al.*, 1996). Raw milk, not fully aseptic conditions during the fermentation process, prophage induction in lysogenic starter strains as well as the environment itself — air, appliances, whey, workers, etc. are commonly regarded as potential sources of bacteriophages (McIntyre *et al.*, 1991). Once having emerged, phages can spread swiftly throughout the dairy plant. Their presence is difficult to eliminate due to their short latent period, relatively large burst size and/or resistance to pasteurization (Daly *et al.*, 1996; Madera *et al.*, 2004). Phage-induced bacterial cell lysis leads to failed or slow fermentation, decrease in acid production, reduction of milk products quality, e.g. taste and texture (Lawrence, 1978; Coffey & Ross, 2002), which all result in profound economical losses.

Lactococcus lactis strains are widely used as starter cultures for milk fermentation during man-

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Abbreviations: pfu, plaque forming units; rcf, relative centrifugation force.

ufacturing of many types of cheeses, sour cream, buttermilk and kefir (Moineau *et al.*, 1996). It is estimated that 60–70% of technological disruptions in production of cottage and hard cheeses is caused by bacteriophage infections of lactococcal strains (refer to Hejnowicz & Bardowski, 2005). Due to its frequent application, many studies have concentrated on bacteriophages active against *L. lactis.* Lactococcal phages most frequently encountered in milk plants belong to one of three types: 936, c2 or P335 (Casey *et al.*, 1993; Moineau *et al.*, 1996; Schouler, 1996). Phages from the P335 type comprise of both temperate and lytic representatives, while c2- and 936-type phages execute only the lytic life cycle (Jarvis *et al.*, 1991).

Phage infections of mesophilic starter cultures in the dairy environment in Poland evoke serious biotechnological problems. Despite this fact, no data is available on the ecology nor molecular characteristics of bacteriophages virulent for *Lactococcus* in our country.

Therefore, the objective of our work was to investigate Polish dairy whey samples in the context of bacteriophage content. It is also the first study of such kind on lactococcal bacteriophages in Poland. Knowledge of the types of phages encountered in milk fermentation environment was expected to supply data on phage biology and biodiversity that could serve for quick phage identification and allow selecting phage-resistant starter strains to overcome phage infections.

MATERIALS AND METHODS

Bacterial strains, bacteriophages and growth conditions. Bacterial strains and bacteriophages used in this study are listed in Table 1. Bacterial strains were cultured at 30°C in M17 broth (Terzaghi & Sandine, 1975) containing 0.5% glucose (GM17) without shaking or on GM17 plates with 1.5% agar. A final concentration of 10 mM of $CaCl_2$ was added to the medium when phage lysates were prepared.

Bacteriophage propagation and purification. Techniques for phage titration and propagation were essentially as described previously (Sambrook *et al.*, 1989) apart from specific modifications as to the medium and incubation temperatures appropriate for *L. lactis*.

Initially, whey samples were analyzed for the presence of phages by separately mixing serial dilutions of the samples with each of the industrial *L. lactis* strains and plating them on double-layer GM17 plates supplemented with 10 mM $CaCl_2$. Phage detection and titer determination were followed by purification of chosen single plaques which was done by reductive streaking using small strips of sterile paper wetted in single phage plaque suspensions on a double-layer lawn of phage-sensitive bacteria.

Small and large scale bacteriophage propagation. Phage propagation was done by small or large scale lysis in liquid medium. Small scale lysis was performed by infecting 1 ml of bacterial culture grown to an OD_{600} 0.2 with purified single plaque picked from the plate by a sterile toothpick. Lysis on a large scale was performed in 200 ml of culture grown to an OD_{600} 0.2 infected with 0.2 ml of the small volume lysate, containing phages at a concentration of approx. 10^8 pfu/ml. A volume of 2 ml from large scale phage lysate was stored at 4°C as a high titer stock.

Isolation of phage DNA. Phage lysates obtained on a large scale were filtered using 0.45 μm filters (Stericup Millipore). RNase and DNase (Sigma) were added to a final concentration of 20 μg/ml each and lysate incubated at 37°C for 1 h. Subsequently, NaCl to a final concentration of 1 M and 10% (w/v) of polyethylene glycol (PEG_{6000}) in powder were added to the lysates and after being completely dissolved by vigorous shaking, stored overnight at 4°C. Phage particle precipitates were recovered by centrifugation at 9000 rcf for 20 min at 4°C and, after discarding the supernatant, suspended in TM buffer (10 mM Tris/HCl, pH 8, 10 mM $MgCl_2$). PEG and cell debris were extracted from phage suspensions by adding chloroform (1:1, v/v) and vigorous vortexing (approx. 30 s). The water phase containing phage particles was recovered after 15 min of centrifugation at 21000 rcf. This was followed by extractions: once with phenol (1:1, v/v) and lithium chloride (1:10, v/v), twice with phenol (1:1, v/v) and once with phenol/chloroform solution (1:1, v/v). Finally, DNA was precipitated from the water phase with cold 96% ethanol (1:2, v/v). DNA was recovered by centrifugation at 21000 rcf for 30 min at 4°C, and washed with 70% ethanol. After drying, the precipitates were dissolved in 100 μl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8) and stored at –20°C.

DNA manipulation and gel electrophoresis. DNA manipulations were performed by standard techniques (Sambrook *et al.*, 1989). Purified phage DNA was digested with *Eco*RI, *Eco*RV, *Hin*dIII and *Hinc*II restriction endonucleases supplied by Boehringer-Mannheim and used as recommended by the manufacturer. Digested DNA samples were analyzed on 0.8% agarose gel containing 0.25 μg/ml ethidium bromide in 1×TAE buffer (0.04 M Tris, 0.04 M acetic acid, 1 mM EDTA, final pH 8.0). Markers-GeneRuler™ 1 kb DNA Ladder (Fermentas) and GeneRuler™ DNA Ladder Mix (Fermentas) were used as relative molecular weight references.

Total DNA isolation from bacterial cells. Total DNA was isolated from *L. lactis* cells by the fol-

Table 1. Bacterial strains and bacteriophages

Bacterial strain or phage		Relevant properties	Reference
***L. lactis* strains**			
Industrial starter strains of *L. lactis* ssp. *lactis* *L. lactis* ssp. *lactis* var. *diacetylactis*, *L. lactis* ssp. *cremoris*		unknown	Rhodia Food Biolacta
L. lactis ssp. *lactis* IL1403		plasmid-free	Chopin, 1984
L. lactis ssp. *cremoris* MG1363		plasmid-free	Gasson, 1983
Bacteriophages			
c2	c2-type representative phage		Pillidge & Jarvis, 1988
712	936-type representative phage		Gasson, 1983
P335	P335-type representative phage		Braun *et al.*, 1989
bIBBg6/5	c2-type		this study
bIBB588p_1	c2-type		this study
bIBB94p_4	c2-type		this study
bIBB94g_5	c2-type		this study
bIBB24g_2	c2-type		this study
bIBB24tp_1	936-type		this study
bIBB5g_1	936-type		this study
bIBBEg_1	936-type		this study
bIBB2a	936-type		this study
bIBB3a	936-type		this study
bIBB5a	936-type		this study
bIBB8a	936-type		this study
bIBB10a	936-type		this study
bIBB40a	936-type		this study
bIBB1	936-type		this study
bIBB5	936-type		this study
bIBB8	936-type		this study
bIBB12	c2-type		this study
bIBB14	c2-type		this study
bIBB18	c2-type		this study
bIBB19	c2-type		this study
bIBB20	c2-type		this study
bIBB22	c2-type		this study
bIBB27	c2-type		this study
bIBB27a	c2-type		this study
bIBB29	936-type		this study
bIBB36	c2-type		this study
bIBB47	936-type		this study
bIBB50	936-type		this study
bIBB55	c2-type		this study
bIBB61	c2-type		this study
bIBB77	c2-type		this study
bIBB89	c2-type		this study
bIBB95	c2-type		this study

lowing method: cells from 2 ml of overnight cultures were harvested, washed and suspended in 0.2 ml of TES-lysozyme solution (25 mM Tris/HCl, 0.1 M EDTA, 25% (w/v) saccharose pH 8, lysozyme 8 mg/ml). After 15 min of incubation at 37°C, 15 µl of 20% SDS solution was added and samples were incubated for 5 min at 75°C. Cell lysates were extracted with 0.5 ml of 1:1 phenol/chloroform solution (1:1, v/v). After centrifugation (15 min, 21000 rcf) the water phase was recovered. The phenol/chloroform extraction was repeated twice. Finally, total DNA was precipitated with 3 M sodium acetate (1:10, v/v, pH 4.8) and ice cold 96% ethanol (1:2, v/v). After centrifugation (15 min, 21000 rcf), the pellet was washed with 70% ethanol, dried and dissolved in 50 µl of demineralized water with RNase (100 µg/ml).

Multiplex PCR. Classification of phages into specific genetic types was performed by multiplex PCR using primers and conditions described elsewhere (Labrie & Moineau, 2000) with 1 µl of either: original whey samples, purified phage lysates or chromosomal DNA of industrial strains as a template. Phages c2, 712 (936-type) and P335 were used as reference phages of the three main genetic types.

Host range assays. To determine the host range of phages, growth of infected lactococcal strain

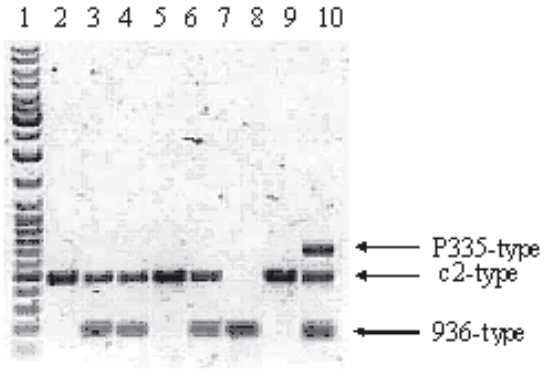

Figure 1. Detection of *L. lactis* phages in whey samples by multiplex PCR.
Primers specific for 936, c2 and P335 phages were used. Lane 1, GeneRuler^{TM} DNA Ladder Mix (Fermentas); lanes 2–9, whey samples (1 μl); lane 10, PCR products obtained from 936-, c2- and P335-type reference phages.

cultures was assayed at 30°C in a Microbiology Reader Analyzer (Bioscreen C; ThermoLabsystems). In the experiment, 4 μl of diluted high titer phage stock (10^8 pfu/ml) was added to 200 μl of bacterial cultures at OD_{600} 0.2, after which the cultures were monitored for at least 12 h and OD_{600} measurements taken every 15 min.

RESULTS

Verification of the presence of bacteriophages in industrial whey samples

Thirty-three whey samples from milk factories located in various regions of Poland were examined for the presence of *L. lactis* phages. Initial screening by multiplex PCR served for a rapid detection of lactococcal phages that belong to the three prevailing lactococcal phage types — c2, 936 and P335. Results of the assay established that phages are unambiguously present in all analyzed wheys (Fig. 1). Among them, 27% wheys contained both c2- and 936-type phages, 30% — only c2-type phages, whereas in 43% of whey samples only 936-type phages were found. Overall, 936-type phages were detected in 23 (69%) of whey samples, which implied that they appear more frequently than c2-type phages.

Individual phages were isolated and purified against 15 *L. lactis* strains selected from a collection of industrial starters. Those strains which exhibited the highest sensitivity towards phage development were used for further phage propagations. In parallel, phage titer in wheys was determined to vary in the range of 10^3–10^{12} pfu/ml, but in the majority of cases it was around 10^9 pfu/ml. High titer suggested that phage-induced lysis was the most plausible factor for the failure of fermentation processes from which the

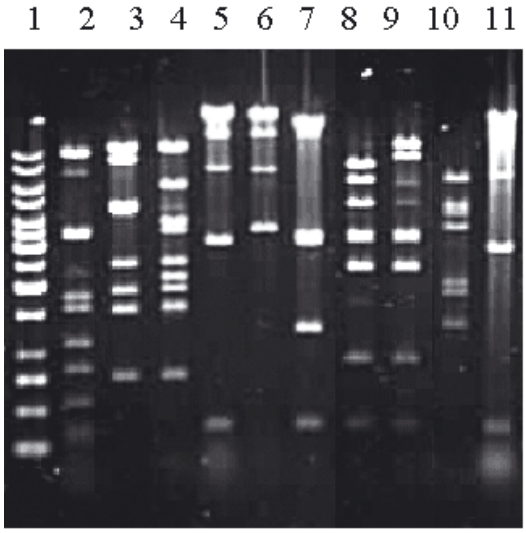

Figure 2. *Eco*RI-restriction DNA patterns of chosen representative phage isolates.
Lane 1, GeneRuler^{TM} 1 kb DNA Ladder (Fermentas); lane 2, bIBB36; lane 3, bIBB47; lane 4, bIBB50; lane 5, bIBB55; lane 6, bIBB61; lane 7, bIBB77; lane 8, bIBB5g$_1$; lane 9, bIBBEg$_1$; lane 10, bIBB89; lane 11, bIBB95.

whey samples had been taken. Based on the morphological features of phage plaques (shape and size) a representative number of plaques (between 5 and 20) from each whey were taken for further examination. As a result, over 200 phage plaques were isolated and purified. Finally, high titer (10^9–10^{11} pfu/ml) phage stocks were prepared by propagating the purified phage plaques in liquid medium. Overall, 223 phage isolates were obtained and subjected to physiological and molecular studies.

Determination of phage DNA restriction patterns

All isolated phages were examined for their restriction patterns with *Eco*RI, *Eco*RV, *Hin*dIII and *Hin*cII. *Eco*RI was determined to be the best in discriminating among the phage DNA samples and was chosen for further digestions (Fig. 2). In effect, 223 phage isolates were grouped into 34 distinct phage restriction groups. Moreover, total phage genome

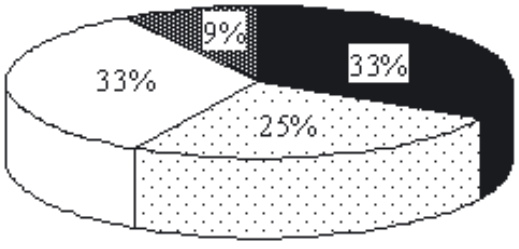

Figure 3. Number of phage DNA restriction groups found in analyzed wheys.
Percentage of wheys containing phages from: one (9), two (25), three (33) or four (33, black) restriction groups.

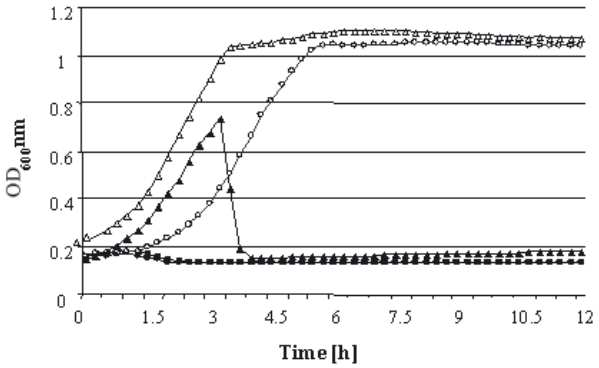

Figure 4. Lytic development of representative bacteriophages on selected *L. lactis* starter strain cultures.
Uninfected *L. lactis* IBB1793B (○), IBB1793B infected with bIBB5g_1 (●), IBB1793B infected with bIBBEg_1 (■), uninfected IBB1784B (Δ), IBB1784B infected with bIBB29 (▲). Strains were grown on GM17 at 30°C until OD_{600} 0.2 after which the cultures were infected at high multiplicity of infection.

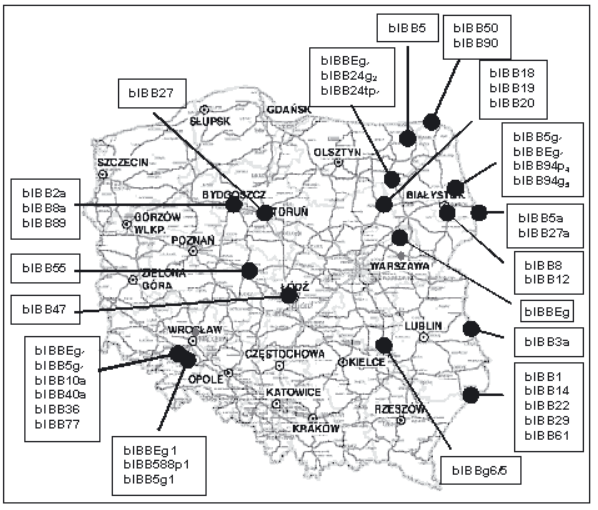

Figure 5. Geographical distribution of virulent lactococcal phages in dairy environment in Poland.
Map shows the regional origin of phage isolates.

sizes were assessed by summing up the molecular sizes of *Eco*RI restriction fragments and determined to range from 14 to 35 kb.

In the majority of wheys phages exhibiting different restriction patterns were detected; 67% of the samples contained phages representing two or more restriction groups (Fig. 3). Among the 34 distinct restriction groups determined, two, represented by phages bIBB5g_1 and bIBBEg_1, were the most prevalent. They were detected, respectively, in 5 and 10 out of the 33 wheys analyzed.

Phage classification into genetic groups of homology

To classify the isolated phages into distinct genetic groups, multiplex PCR analysis was performed using primers specific for detecting DNA homology to c2-, 936- or P335-type phages. Among the phages with distinct restriction patterns determined an equal distribution of c2- (17) and 936-type (17) phages was shown, in contrast to P335-type phages which were not detected at all. The most prevalent bacteriophages — bIBB5g_1 and bIBBEg_1 — were classed as 936 type. To further investigate the lack of P335-type phages in the whey samples, multiplex PCR was performed using type-specific primers on total DNA extracts from industrial starter strains. Notably, in 75% of the strains tested a product specific for P335-type phages was detected (not shown). Such a result suggested presence prophages of P335-type in chromosomes of the majority of the starter strains.

Dynamics of infection and phage host range assays

To determine the host range and dynamics of infection representative phages from each restriction group were tested against 15 different *L. lactis* ssp. *lactis*, *L. lactis* ssp. *lactis* var. *diacetylactis*, *L. lactis* ssp. *cremoris* industrial starter strain cultures. Four strains, namely IBB1783B, IBB1788B, IBB1794B and IBB1800B, were resistant to all of the examined phages (Table 2). Otherwise, lysis of nearly all phage-sensitive bacterial cultures occurred approximately 2 h after infection which implied a rather fast phage infection rate (Fig. 4). Among them, four strains appeared highly susceptible to phage infections and were lysed by representative phages from the c2- as well as the 936-type. Most of the phages, representative of all restriction groups, demonstrated a narrow host range. Phages representatives of 23 out of 34 restriction groups were active against only one strain; phage representatives from 9 other restriction groups were active against two strains, while only two phage representatives from the remaining groups lysed three strains. Phages belonging to the c2-type displayed a wider host range than 936-type phages. Notably, 41% of c2-type phages studied infected more than two strains, while the majority of the identified 936-type phages were lytic for only one strain. Two 936-type phages most commonly detected — bIBB5g_1 and bIBBEg_1 — were active on only one strain.

Analysis of the geographical distribution of the isolated dairy bacteriophages

The biodiversity of wheys supplied from 17 different regions in Poland was studied in respect to the region of origin by examining their phage content (Fig. 5). In all wheys at least one original phage was present. Phages prevalent in several regions were also identified — the most frequently appear-

Table 2. ***L. lactis*** **strain sensitivity towards isolated phages**

Phage	Phage type	Bacterial strain														
		IBB 094	IBB 1767B	IBB 1772B	IBB 1780B	IBB 1782B	IBB 1783B	IBB 1784B	IBB 1787B	IBB 1788B	IBB 1791B	IBB 1793B	IBB 1794B	IBB 1796B	IBB 1800B	IBB 1807B
bIBBg6/5	c2															+
bIBB588p_1	c2													+		
bIBB94p_4	c2	+														
bIBB94g_5	c2	+														
bIBB24g_2	c2											+				
bIBB24tp_1	936											+				
bIBB5g_1	936											+				
bIBBEg_1	936											+				
bIBB2a	936								+							
bIBB 3a	936								+							
bIBB 5a	936								+							
bIBB 8a	936											+				
bIBB 10a	936											+				
bIBB 40a	936											+				
bIBB 1	936				+						+					
bIBB 5	936				+						+					
bIBB 8	936				+						+					
bIBB 12	c2								+							
bIBB 14	c2							+						+		
bIBB 18	c2							+						+		
bIBB 19	c2							+						+		
bIBB 20	c2							+						+		
bIBB 22	c2							+						+		
bIBB 27	c2				+			+						+		
bIBB 27a	c2													+		
bIBB 29	936							+				+		+		
bIBB 36	c2			+												
bIBB 47	936				+											
bIBB 50	936				+											
bIBB 55	c2															+
bIBB 61	c2					+										+
bIBB 77	c2					+										
bIBB 89	c2		+													
bIBB 95	c2													+		

ing phages — bIBB5g_1 and bIBBEg_1 — were found respectively in three and seven out of 17 analyzed areas. Yet, our observations show that the majority of phages seem to be specific to geographical regions. Most probably this is directly due to the starter strains used in the dairy plants.

DISCUSSION

This work provides novel data on lactococcal bacteriophages in Poland. Phage infections of the mesophilic lactococcal starters constitute a serious biotechnological problem in Polish dairy plants. Yet, no details about their molecular features or ecology are available. Our 2-year study resulted in establishing a wide collection of lactococcal lytic bacteriophages isolated from the dairy environment. Basic molecular genetic techniques allowed determining such characteristics as restriction patterns, genome sizes, genetic similarities and host ranges. We determined that all wheys contained phages at a high titer which implied that phage infections were the most certain cause of fermentation perturbations.

The range of phages detected in the analyzed whey samples reflects the great diversity of phages that persist in dairy plants. A comparison of DNA restriction patterns revealed a number of similarities among the studied phages that suggest either partial conservation of phage genetic content or events of extensive DNA recombination that occur between them. Genetic type classification assays grouped the phages detected

in wheys into either c2 or 936 types, which comprise phages that execute only the lytic cycle. This excluded the possibility that the isolated phages could originate from lysogenic starter cultures, although the majority of industrial host strains used in this study contain prophages of P335-type in their chromosomes. The observed lysogenicity of the strains is in agreement with the results from previous studies which showed that lactococcal starter culture strains are commonly found to carry prophages (Huggins & Sandine, 1977; Gasson, 1980; Terzaghi & Sandine, 1981; Reyrolle *et al.*, 1982). The lack of P335-type phages in the analyzed whey samples is also consistent with the idea that lytic phages from this type appeared in the dairy environment only recently (Moineau *et al.*, 1993). Nevertheless, even if an induction of prophages of P335-type occurred, they were undetectable in the wheys under the conditions of the multiplex PCR assay. This suggests that P335-type phages constituted a small minority, if at all, of phage particles present in the examined wheys and could not have been the cause of biotechnological problems.

In our study, no correlation between plaque morphology and phage DNA restriction pattern was observed. Phages from different restriction and genetic groups were noted to be able to propagate in the same host, which implies that there is no relation between DNA homology and host specificity.

Lactococcal phages identified in this work were found to belong to either the c2 or 936 types of lytic phages. Yet, the frequencies at which the two phage types were isolated differed — 936-type phages appeared more commonly than c2-type phages. This observation is consistent with reports from other European countries as well as from New Zealand, United States or Canada, where the lytic lactococcal 936-type phages were found to dominate (Jarvis, 1984; Prevots *et al.*, 1990; Casey *et al.*, 1993; Moineau *et al.*, 1996; Bissonnette *et al.*, 2000; Miklic & Rogelj, 2003). Notably, a different situation was reported in countries localized nearby Poland. In Germany, 60% of the analyzed phages were classified as c2-type, while a similar study in Denmark established the prevalence of phages from the P335 type (Lembke *et al.*, 1980; Josephsen *et al.*, 1999). Earlier publications show that the type of the isolated phages is correlated with bacterial strains used in starter culture mixes (Jarvis, 1989; Daly *et al.*, 1996). It has been noted that starter cultures containing defined strains, mainly *L. lactis* subsp. *cremoris,* favor isometric-headed phage propagation, including the 936 type, while mix starter cultures contain an excess of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *lactis* var. *diacetylactis* strains, which are hosts for c2-type prolate-headed phages. Based on our observations, a preference for defined starter cultures in dairy plants in Poland can be suggested.

Most cultures monitored were lysed after 2 h following infection, indicating a fast phage lysis rate. The majority of the studied phages exhibited a narrow host spectrum. The c2-type phages isolated in this study displayed a wider host range than the 936-type phages. This observation is consistent with earlier reports stating that c2-type phages are less specific to bacterial strains and demonstrate a large host spectrum (Heap & Jarvis, 1980; Lodics & Steenson, 1990; Moineau *et al.*, 1992; 1996; Miklic & Rogelj, 2003). Yet, notably, the two most prevalent phages — bIBB5g_1 and bIBBEg_1 — which each infected only one *L. lactis* subsp. *lactis* var. *diacetylactis* starter strain, were found to belong to the 936 type. The common occurrence of these two phages in various dairy plants is most likely connected with the frequent exploitation of the sensitive bacterial strains throughout the whole country and specialization of the predominant phages towards these two strains. Strains employed in this study have been selected among potentially phage-resistant bacteria used as industrial starters, which supports the fact that almost 94% of the phages were specific for only one or two bacterial strains and four strains were resistant to all phages tested. Yet, the fast lysis rate and narrow host range reflect phage specialization to the starter strains exploited in the dairy environment. On the other hand, certain strains are sensitive to both c2- and 936-type phages, which suggests that phages of different types have independently developed an adaptation of infecting the same strain.

Analysis of the actual state of phages predominant in the dairy industry in Poland shows that lactococcal phages prevail and are still a common cause of fermentation perturbations. The incidence of phage infections in milk plants rises with the worldwide development of this industrial branch, a direct result of the increase of the amount of processed milk. Our collection of industrial starter cultures and lactococcal phages isolated from industrial whey samples supplies a valuable reference on phage-resistant starter cultures and phages most commonly occurring in dairies. Elimination or reduction of phage infection incidences depend highly on the multiple strain starters used. Successful employment of starter bacteria is associated with a number of factors; primarily, with selecting strains with relatively low phage-sensitivity that will be able to endure the attack of phages. Therefore, a wider knowledge of phages present in dairy plants as well as of their biology seems indispensable.

Acknowledgements

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