Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Development of a species-specific PCR assay for identification of the strictly anaerobic bacterium *Selenomonas lacticifex* found in biofilm-covered surfaces in brewery bottling halls

J. Felsberg[1], M. Jelínková[1], P. Kubizniaková[2] and D. Matoulková[2]

1 Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic
2 Department of Microbiology, Research Institute of Brewing and Malting, Prague, Czech Republic

Keywords
beer spoilage, biofilm, PCR, secondary contamination, *Selenomonas lacticifex*, strictly anaerobic bacteria.

Correspondence
Dagmar Matoulková, Department of Microbiology, Research Institute of Brewing and Malting, Lípová 15, 120 44 Prague, Czech Republic.
E-mail: matoulkova@beerresearch.cz

2014/1324: received 29 June 2014, revised 16 July 2014 and accepted 24 July 2014

doi:10.1111/jam.12610

Abstract

Aims: In recent years, beer-spoilage cases from strictly anaerobic bacteria have risen in frequency, in connection with the production of non-pasteurized, non-alcohol and low-alcoholic beers and with the lowering of dissolved oxygen in the packaged beer. *Selenomonas lacticifex*, found in brewer's yeast and in biofilms covering some surfaces in brewery bottling area, is considered to be a beer-spoilage organism. This study aims to develop *S. lacticifex*-specific PCR assay. The objective of this study was also evaluation of the specificity and reproducibility of the developed PCR assay in real brewery samples.
Methods and Results: Three primers (one forward and two reverse) were designed for identification of the strictly anaerobic bacterium *S. lacticifex* on the basis of the species-specific sequences of the 16S rDNA region. The specificity of the primers was tested against 44 brewery-related non-target micro-organisms that could potentially occur in the same brewery specimens. None of the primer pairs amplified DNA from any of the non-*S. lacticifex* strains tested including genera from the same family (*Pectinatus*, *Megasphaera*, *Zymophilus*) and the closely related species *Selenomonas ruminantium*, showing thus 100% specificity.
Conclusions: The PCR assay developed in this study enables the detection of the strictly anaerobic bacterium *S. lacticifex* in real brewery samples including pitching yeast.
Significance and Impact of the Study: *Selenomonas lacticifex*-specific PCR assay developed in this study allows for the extension of the spectra of detected beer-spoilage micro-organisms in brewing laboratories and thus lowering the risk of contamination of the final product.

Introduction

The current trend in the bottling technologies leads to the lowering of oxygen tension in finished beer to a minimum (below 1 mg l^{-1}). Beer thus becomes a medium in which strictly anaerobic bacteria can thrive (Back 2005). Strictly anaerobic bacteria found in connection with the brewery environment fall within four genera: *Pectinatus*, *Zymophilus*, *Selenomonas* and *Megasphaera*. These genera are currently classed into the family *Veillonellaceae* (phylum Firmicutes, class Negativicutes, order Selenomonadales).

The genus *Selenomonas* is mostly associated with oral cavity of humans, the microflora of the rumen of herbivores and the intestine of pigs and some rodents (Kingsley and Hoeniger 1973; Schleifer *et al.* 1990; Ricke *et al.* 1996; Shouche *et al.* 2009). However, one of the existing 11 species, *Selenomonas lacticifex*, occupies a diverse

environment—pitching yeast (Haikara 1989). *Selenomonas* cells are typically moon-like to helical, with some flagella originating from a bunch or a short line in the central part of the concave side of the cell. The cells occur singly, in pairs or in short chains. The motion of *Selenomonas* can be described as tumbling (Kingsley and Hoeniger 1973; Chelack and Ingledew 1987; Schleifer *et al.* 1990). *Selenomonas lacticifex* ferments glucose to lactic acid as the main product and acetic and propionic acids as side products (Schleifer *et al.* 1990).

Selenomonas lacticifex is considered to be a beer-spoilage organism, although it has never been isolated directly from beer. When purified from contaminated brewer's yeast, it was able to grow in artificially contaminated beer (Seidel-Rüfer 1990; Vaughan *et al.* 2005; Matoulková 2008). Its occurrence was exclusively linked with brewer's yeast in modern brewery plants; however, the presence of *S. lacticifex* was recently revealed in biofilms covering some surfaces in brewery bottling area (Vávrová *et al.* 2014). Bacteria embedded in the biofilm can be transmitted to fillers and subsequently to packaged beer via aerosol generated during the filling process and cleaning procedures (Paradh *et al.* 2011; Suzuki 2011). The presence of this bacterium close to the filling machine may thus signify a high risk for packaged beer, especially in modern brewery plants producing beers with very low oxygen content.

The presence of *Selenomonas* spp. in a brewery escapes conventional microbiological checks because no specific diagnostic culture media exist. Nonselective modified de Man–Rogosa–Sharpe (MRS) medium (Schleifer *et al.* 1990), thioglycollate medium (Motoyama and Ogata 2000; Asano *et al.* 2008) and PYF (peptone yeast extract-fructose) broth (Juvonen *et al.* 2008) are used for cultivation of collection strains of *Selenomonas* spp., with incubation at 27–30°C under anaerobic conditions. However, the media are not selective and do not enable specific identification of *Selenomonas*.

A group-specific PCR-RFLP and real-time PCR methods were developed for detection of bacteria *Pectinatus*, *Megasphaera*, *Selenomonas* and *Zymophilus* in beer. Genus *Selenomonas* and other genera in the group-specific endpoint and real-time PCR products can be further differentiated using restriction fragment length polymorphism (*ScaI*) and melting point curve analysis, respectively (Juvonen *et al.* 2008). Recently, the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-MS) analysis was successfully applied for direct identification of beer contaminants including the species *S. lacticifex* (Vávrová *et al.* 2014).

The aim of this study was to design *S. lacticifex*-specific primers based on the 16S ribosomal RNA gene region that would enable us to detect *S. lacticifex* in a single end-point or real-time PCR reaction with no downstream processes needed.

Materials and methods

Micro-organisms and culture conditions

The strains used in this study were obtained from the Czech Collection of Micro-organisms (CCM), German Collection of Micro-organisms and Cell Cultures (DSMZ), VTT Culture Collection of Technical Research Centre of Finland (VTT) and Collection of Research Institute of Brewing and Malting (RIBM). A total of 47 strains including 3 strains of *S. lacticifex*, ruminal strain *Selenomonas ruminantium* DSM 2150, *Pectinatus brassicae* DSM 24661, 40 strains of bacteria that can occur as a potential contamination of finished beer, pitching yeast and the various stages of malting and brewing process, and 2 strains of culture brewer's yeast strains (ale yeast *Saccharomyces cerevisiae* and lager yeast *S. pastorianus*) were used in the study. Strains, culture media and conditions of cultivation are listed in Table 1. Modified MRS broth contained cysteine hydrochloride (Sigma-Aldrich, Prague, Czech Republic) and sodium thioglycollate (Sigma-Aldrich), both in a concentration of 0·25 g l^{-1}, pH 5·6–5·8. Nutrient agar was purchased from Merck (Merck, Prague, Czech Republic). YPD agar was prepared from yeast extract (10 g l^{-1}, Oxoid, Brno, Czech Republic), peptone (20 g l^{-1}, Oxoid), glucose (20 g l^{-1}, Merck) and bacteriological agar (20 g l^{-1}, Oxoid).

DNA preparation

The DNA was extracted using NucleoSpin Tissue Kit (Macherey Nagel, Dueren, Germany) according to manufacturer's instructions and stored in TE-buffer at 4°C.

Design of the species-specific primers

The forward primer 5′-AGA-GTT-TGA-TCC-TGG-CTC-AG-3′ and reverse primer 5′-ACG-GCA-ACC-TTG-TTA-CGA-GTT-3′ according to Satokari *et al.* (1998) and Doyle *et al.* (1995), respectively, were used for amplification of whole 16S rDNA gene of six strictly anaerobic bacteria found in connection with brewery contamination. Thermocycler TGradient (Biometra, Göttingen, Germany) was used for all DNA amplification assays. PCR was set up in a 50-μl reaction volume containing 25 μl of Combi PPP Master Mix (Top-Bio, Prague, Czech Republic), 20 μl of PCR-H_2O, 2 μl of each primer (10 μmol l^{-1}) and 1 μl of DNA (50–100 ng). Initial denaturation was performed at 95°C for 5 min, followed by 39 amplification cycles. The amplification profile was

Table 1 Micro-organisms, media and incubation conditions, and results of PCR identification

Strain*	Media/incubation†	PCR with primers SelenoFor2/ SelenoRev1	PCR with primers SelenoFor2/ SelenoRev2
Selenomonas lacticifex DSM 20757	mMRS broth/Anaerobic at 28°C	+	+
S. lacticifex VTT E-86269	mMRS broth/Anaerobic at 28°C	+	+
S. lacticifex VTT E-86273	mMRS broth/Anaerobic at 28°C	+	+
Selenomonas ruminantium DSM 2150	mMRS broth/Anaerobic at 28°C	–	–
Pectinatus cerevisiiphilus DSM 20467	mMRS broth/Anaerobic at 28°C	–	–
Pectinatus frisingensis DSM 20465	mMRS broth/Anaerobic at 28°C	–	–
P. frisingensis RIBM 2-86	mMRS broth/Anaerobic at 28°C	–	–
Pectinatus haikarae DSM 16980	mMRS broth/Anaerobic at 28°C	–	–
Pectinatus brassicae DSM 24661	mMRS broth/Anaerobic at 28°C	–	–
Megasphaera cerevisiae DSM 20461	mMRS broth/Anaerobic at 28°C	–	–
M. cerevisiae DSM 20462	mMRS broth/Anaerobic at 28°C	–	–
Megasphaera paucivorans DSM 16981	mMRS broth/Anaerobic at 28°C	–	–
Zymophilus paucivorans DSM 20756	mMRS broth/Anaerobic at 28°C	–	–
Zymophilus raffinosivorans DSM 20765	mMRS broth/Anaerobic at 28°C	–	–
Acetobacter aceti CCM 3620^T	YPD agar/Aerobic at 28°C	–	–
Acetobacter pasteurianus CCM 2374^T	YPD agar/Aerobic at 26°C	–	–
Bacillus cereus CCM 2010^T	Nutrient agar/Aerobic at 28°C	–	–
Citrobacter freundii CCM 7187	Nutrient agar/Aerobic at 37°C	–	–
Enterobacter aerogenes CCM 7797^T	Nutrient agar/Aerobic at 37°C	–	–
Enterococcus faecalis CCM 7000^T	Nutrient agar/Aerobic at 37°C	–	–
Escherichia coli CCM 5172^T	Nutrient agar/Aerobic at 37°C	–	–
Gluconobacter oxydans CCM 3607^T	YPD agar/Aerobic at 26°C	–	–
Hafnia alvei CCM 4845	Nutrient agar/Aerobic at 28°C	–	–
Klebsiella oxytoca CCM 3565	Nutrient agar/Aerobic at 37°C	–	–
Kocuria kristinae CCM 2690^T	Nutrient agar/Aerobic at 37°C	–	–
Lactobacillus brevis RIBM 2-16	MRS agar/Anaerobic at 28°C	–	–
Lactobacillus brevis RIBM 2-56	MRS agar/Anaerobic at 28°C	–	–
Lactobacillus buchneri CCM 1819^T	MRS agar/Anaerobic at 37°C	–	–
Lactobacillus casei/paracasei RIBM 2-88	MRS agar/Anaerobic at 28°C	–	–
Lactobacillus casei/paracasei RIBM 2-95	MRS agar/Anaerobic at 28°C	–	–
Lactobacillus curvatus CCM 7558^T	MRS agar/Anaerobic at 37°C	–	–
Lactobacillus plantarum RIBM 2-29	MRS agar/Anaerobic at 28°C	–	–
Lactobacillus plantarum RIBM 2-89	MRS agar/Anaerobic at 28°C	–	–
Leuconostoc mesenteroides CCM 2086^T	MRS agar/Anaerobic at 28°C	–	–
Micrococcus luteus CCM 169^T	Nutrient agar/Aerobic at 28°C	–	–
Obesumbacterium proteus CCM 2805	Nutrient agar/Aerobic at 30°C	–	–
Pantoea agglomerans CCM 3490	Nutrient agar/Aerobic at 28°C	–	–
Pediococcus damnosus CCM 3453^T	MRS agar Anaerobic at 28°C	–	–
Pediococcus inopinatus CCM 3451^T	MRS agar Anaerobic at 28°C	–	–
Pediococcus pentosaceus CCM 7796^T	MRS agar Anaerobic at 28°C	–	–
Rahnella aquatilis CCM 4086	Nutrient agar Aerobic at 37°C	–	–
Saccharomyces cerevisiae RIBM 138	YPD agar/Aerobic at 26°C	–	–
Saccharomyces pastorianus RIBM 95	YPD agar/Aerobic at 26°C	–	–
Salmonella enterica CCM 7933^T	Nutrient agar Aerobic at 37°C	–	–
Serratia marcescens CCM 303^T	Nutrient agar Aerobic at 37°C	–	–
Shigella flexneri CCM 4422	Nutrient agar Aerobic at 37°C	–	–
Zymomonas mobilis CCM 2770	YPD agar/Aerobic at 28°C	–	–

*Strain numbers: CCM—Czech Collection of Micro-organisms, Faculty of Science, Masaryk University, Brno, Czech Republic; DSM—German Collection of Micro-organisms and Cell Cultures, Braunschweig, Germany; RIBM—Collection of The Research Institute of Brewing and Malting, Department of Microbiology, Prague, Czech Republic; VTT—Culture Collection, Technical Research Centre of Finland, Espoo, Finland.
†mMRS: modified MRS broth containing cysteine hydrochloride and sodium thioglycollate, both in a concentration of 0·25 g l^{-1}, pH 5·6–5·8.

95°C for 30 s (denaturation), 55°C for 30 s (annealing) and 72°C for 90 s (extension). Final extension was carried out at 72°C for 5 min; PCR reaction was terminated at 4°C. PCR products of 1·5 kb size were then purified using MinElute PCR Purification Kit (Qiagen, Hilden, Germany). The product was then analysed by gel electrophoresis using 1·0% agarose in 1× TAE buffer and by ethidium bromide staining. Detection was carried out using BioDocAnalyze documentation system (Biometra). Products were stored in TE-buffer at −20°C. Purified PCR fragments were sequenced with the ABI PRISM BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). The chain termination reaction was performed in a cycle sequencing technique according to manufacturer′s protocol. Sequences were determined with an ABI PRISM 3130*xl* DNA sequencer (Applied Biosystems). All these six sequences of 16S rDNA were edited and compared using the ClustalW method. Three *S. lacticifex*-specific primers—one forward and two reverse—were designed from this comparative analysis. Regions with the greatest differences in the nucleotide sequences were selected as binding sites for the *Selenomonas*-specific primers. In particular, the differences at the primers′ 3′-end are important for the specificity of the amplification reaction. The respective sequences of these primers are listed in Table 2.

PCR amplification and conditions

All oligonucleotide primers used in this study were synthesized by Sigma-Aldrich. 824-bp and 850-bp fragments of *S. lacticifex* 16S rRNA gene were amplified using the forward primer SelenoFor2 and reverse primers SelenoRev1 and SelenoRev2, respectively, in separate reactions. PCR assay was set up in a 50-μl reaction volume containing 25 μl of Combi PPP Master Mix (Top-Bio), 20 μl of PCR H_2O, 2 μl of each primer (10 μmol l^{-1}) and 1 μl of DNA (50–100 ng). Initial denaturation was performed at 94°C for 60 s, followed by 35 amplification cycles. The amplification profile was 94°C for 30 s (denaturation), 68°C for 10 s (annealing) and 72°C for 90 s (extension). Final extension was carried out at 72°C for 5 min; PCR reaction was terminated at 4°C. The amplification products were analysed by gel electrophoresis using 1·0% agarose in 1× TAE buffer and by ethidium bromide staining. Detection was carried out using video documentation system BioDocAnalyze (Biometra).

Table 2 PCR primers designed in this study

Name	Oligonucleotide sequence (5′ → 3′)
SelenoFor2	CGG GGA CGA ATG TGC AGT ATT T
SelenoRev1	GGC TTC GCT GCT CTC TGT CCA
SelenoRev2	GGT TTA TGG GGT TCG CTT GG

Brewery plant sampling

Samples were collected from the biofilm-covered surfaces after the monoblock (filling machine and crown-cork machine situated in a monoblock arrangement) in the brewery bottling area. Sampling sites were as follows: plastic sleeves, bundles of cable lines underneath the conveyor belt, conveyor belt construction stand, driving rollers underneath the conveyor belt and stainless steel conveyor belt cover—inside part and side ledge of conveyor belt cover. Collection of samples was carried out using sterile cotton wool sticks wetted immediately before sampling with sterile distilled water containing 0·25 g l^{-1} cysteine hydrochloride and 0·25 g l^{-1} sodium thioglycollate. Immediately after sampling, the stick was placed in a test tube with modified MRS-T medium that contained actidione (25 mg l^{-1}; Sigma-Aldrich) for inhibition of yeasts and a mixture of tetrahydroisoalpha-acids (50 mg l^{-1}; Yakima Chief Inc., Sunnyside, WA) for inhibition of Gram-positive bacteria, with pH adjusted to 7·0, according to Matoulková *et al.* (2012a). MRS-T medium was developed primarily for detection of *Pectinatus*; its adjusted composition, however, enables also the growth of *Selenomonas*—for this purpose, the 2-phenylethanol and agar used in original MRS-T medium were omitted. Cultivation of samples was performed anaerobically at 28°C for up to 72 h. The haze of the samples was checked at 12-h intervals. Samples were then checked microscopically, and those containing moon-like cells of putative *S. lacticifex* were used for DNA extraction. Prior to analyses, the DNA was stored as described above.

DNA sequencing and analysis

Purified PCR fragments were sequenced with the ABI PRISM BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems). The chain termination reaction was performed in a cycle sequencing technique according to manufacturer's protocol. Sequences were determined with an ABI PRISM 3130*xl* DNA sequencer (Applied Biosystems).

Results

Design of *Selenomonas lacticifex*-specific primers

The forward primer SelenoFor2 and reverse primers SelenoRev1 and SelenoRev2 were designed on the basis of

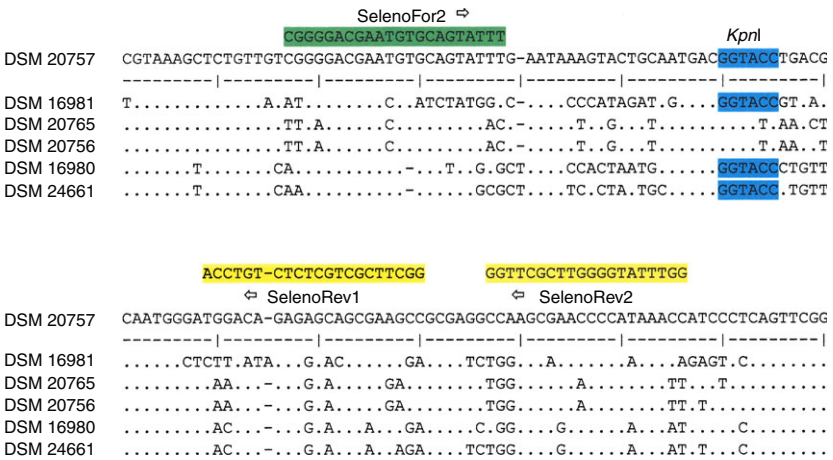

Figure 1 Parts of the alignment of genes coding for the 16S rRNA molecules of *Selenomonas lacticifex* and related bacteria. The positions of the designed primers SelenoFor2, SelenoRev1 and SelenoRev2 in the same way as their direction of action are indicated. The sequences of the unique restriction site for the KpnI endonuclease are shown in boxes. Strains: DSM 20757 – *S. lacticifex*, DSM 16981 – *Megasphaera paucivorans*, DSM 20765 – *Zymophilus raffinosivorans*, DSM 20756 – *Zymophilus paucivorans*, DSM 16980 – *Pectinatus haikarae*, DSM 24661 – *Pectinatus brassicae*.

regions with the greatest differences in the nucleotide sequences of the 16S rDNA region of *S. lacticifex* and other five related strictly anaerobic bacteria (Fig. 1).

Performance characteristics of the PCR assay

Performing PCR reactions with the use of usual annealing temperature (55–60°C) yielded a product with all *Selenomonas*, *Pectinatus*, *Megasphaera* and *Zymophilus* strains used in the study. The optimal annealing temperature of both pairs of primers was tested by gradient PCR with annealing temperature from 60 to 70°C (results not shown). The optimal annealing temperature for identification of *S. lacticifex* (of both pairs of primers), as determined by gradient PCR (data not shown), was 68°C.

Sensitivity and specificity of the PCR assay

The specificity of the designed forward primer SelenoFor2 and the two reverse primers SelenoRev1 and SelenoRev2 was assessed using DNA from a variety of strains belonging to the same family (1 strain of *S. ruminantium*, 5 strains of *Pectinatus*, 3 strains of *Megasphaera*, 2 strains of *Zymophilus*) and of other micro-organisms that could occur in the same brewery specimens (lactic acid bacteria, acetic bacteria, *Enterobacteriaceae*, brewer's yeast etc.), in a total of 44 strains (Table 1). The use of primers SelenoFor2 and SelenoRev1 in PCR reaction with *S. lacticifex* DNA allowed for obtaining one amplicon of 824-bp size. With the primer pair SelenoFor2 and SelenoRev2, even in optimized assay conditions, a weaker band of 850 bp was observed (Fig. 2). None of the primer pairs amplified DNA from any of the non-*S. lacticifex* strains tested, showing thus 100% specificity. Nor was amplified the DNA of *S. ruminantium* that was chosen as the non-brewery representative of the genus *Selenomonas*.

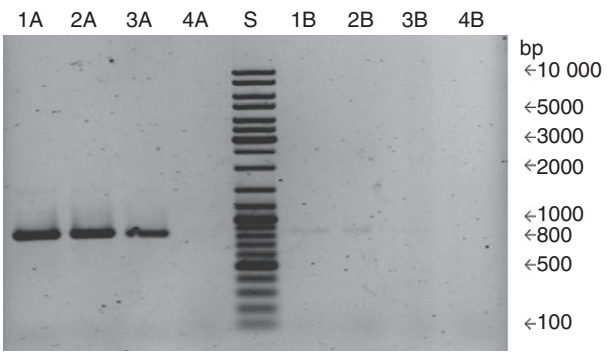

Figure 2 Specificity and sensitivity test of the SelenoFor2+ SelenoRev1 primers (A) and SelenoFor2+ SelenoRev2 primers (B) with the genomic DNA of *Selenomonas lacticifex* and *Selenomonas ruminantium*. The PCR reaction products were electrophoresed on 1·0% agarose gel. 1, *S. lacticifex* DSM 20757; 2, *S. lacticifex* VTT E-86269; 3, *S. lacticifex* VTT E-86273; 4, *S. ruminantium* DSM 2150; S, size marker (GeneRuler DNA Ladder Mix #SM0332, Thermo Fisher Scientific Inc., Waltham, MA).

Analysis of real brewery samples

The developed PCR assay was used for detection of putative *S. lacticifex* from brewery environment. Samples were taken from a bottling line in a middle-sized brewery plant. All samples were collected from the biofilm-covered surfaces after monoblock in the bottling hall. The haze of the samples was checked at 12-h intervals. Samples with cloudy haze at the bottom of the media column (Fig. 3) were then checked microscopically, and those containing moon-like cells (Fig. 4) of putative *S. lacticifex* were used for DNA extraction and PCR assay with the designed primers SelenoFor2, SelenoRev1 and SelenoRev2. In all selected samples, both 824- and 850-bp fragments were separately amplified. These PCR fragments were sequenced with the ABI PRISM BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems).

Figure 3 Example of typical cloudy haze at the bottom of the media column caused by the growth of *Selenomonas lacticifex*. Incubation was performed at 28°C for 48 h.

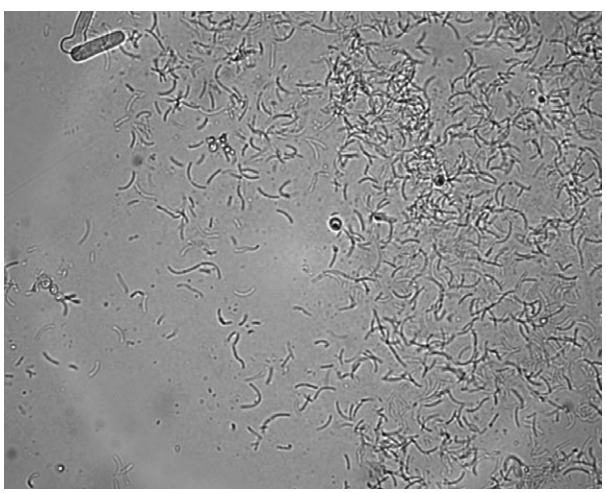

Figure 4 Moon-like cells of *Selenomonas lacticifex* in a sample taken from the biofilm-covered surface of the bundles of cable lines underneath the conveyor belt after the monoblock. Evaluation after 48 h of cultivation in modified de Man–Rogosa–Sharpe medium at 28°C. Magnification ×630.

The results of the sequencing showed that DNA came from *S. lacticifex* bacterium.

Discussion

The phenotypic identification of *S. lacticifex* is difficult and time-consuming, and specific media or molecular diagnostic tests are not yet available. A group-specific PCR-RFLP and real-time PCR methods for simultaneous detection of bacteria *Pectinatus*, *Megasphaera*, *Selenomonas* and *Zymophilus* were developed by Juvonen *et al.* (2008). Genus *Selenomonas* can be further differentiated using restriction fragment length polymorphism (*ScaI*) by end-point PCR, or with melting point curve analysis in real-time PCR arrangement. MALDI-MS analysis was recently successfully applied for direct identification of beer contaminants (Vávrová *et al.* 2014). However, these methods have some disadvantages making their use limited in brewing practice—high acquisition cost (Real-time PCR, MALDI), or an increased risk of laboratory cross-contamination (PCR-RFLP). In the present study, we developed an end-point PCR assay for identification of *S. lacticifex* based on the 16S rDNA region that is 100% species specific and no further step is needed.

Comparison of sequences of whole 16S r DNA gene of *S. lacticifex* DSM 20757, *Megasphaera paucivorans* DSM 16981, *Zymophilus raffinosivorans* DSM 20765, *Zymophilus paucivorans* DSM 20756, *Pectinatus haikarae* DSM 16980 and *P. brassicae* DSM 24661 showed high similarity. The designed primers, forward primer SelenoFor2 and reverse primers SelenoRev1 and SelenoRev2, are based on the species-specific sequences of the 16S rDNA region of *S. lacticifex* and differ only in several nucleotides from other strains (Fig. 1). Melting temperature of all three designed primers is very high due to high GC content. The primer pair SelenoFor2/SelenoRev1 consistently provided greater sensitivity than SelenoFor2/SelenoRev2 (as documented in Fig. 2) and was therefore selected as the optimal candidate for identifying *S. lacticifex* species.

Under the optimized PCR conditions, the primers specificity and reproducibility were evaluated for both primer pairs separately, using a diverse collection of strains including lactic acid bacteria (*Lactobacillus*, *Pediococcus*, *Leuconostoc*), acetic acid bacteria, bacteria of the family *Enterobacteriaceae* and others (Table 1), that could be found in brewery plant environment including also areas with potential formation of biofilms (Henriksson and Haikara 1991; Back 1994; Timke *et al.* 2005; Juvonen and Suihko 2006; Storgards *et al.* 2006; Paradh *et al.* 2011; Matoulková *et al.* 2012b; Bokulich and Bamforth 2013). There was 100% sensitivity and specificity of both pairs of primers—no DNA from any of the

non-*S. lacticifex* strains was amplified (Table 1). As no amplification products were obtained with *Saccharomyces* (both ale and lager yeast) DNA, the PCR assay developed in this study can be also used for identification of *S. lacticifex* contamination in pitching yeast. As was recently published by Vávrová *et al.* (2014), *S. lacticifex* can be found in brewery bottling hall environment in association with biofilm. In this study, we have confirmed the presence of the strictly anaerobic bacterium *S. lacticifex* in real brewery samples taken from the biofilm-covered surfaces.

Acknowledgements

This work was supported by the Institutional Research Concept RVO61388971 and by the Grant Agency of the Czech Republic (GAP503/12/1424).

Conflict of Interest

No conflict of interest to declare.

References

Asano, S., Suzuki, K., Kuriyama, H., Yamashita, H. and Kitagawa, Y. (2008) Application of multiplex PCR to the detection of beer spoilage bacteria. *J Am Soc Brew Chem* **66**, 37–42.

Back, W. (1994) Secondary contaminations in the filling area. *Brauw Int* **4**, 326–334.

Back, W. (2005) Brewery. In *Colour Atlas and Handbook of Beverage Biology* ed. Back, W. pp. 10–112. Nürnberg, Germany: Verlag Hans Carl.

Bokulich, N.A. and Bamforth, C.W. (2013) The microbiology of malting and brewing. *Microbiol Mol Biol Rev* **77**, 157–172.

Chelack, B.J. and Ingledew, W.M. (1987) Anaerobic gram-negative bacteria in brewing – a review. *J Am Soc Brew Chem* **45**, 123–127.

Doyle, L.M., McInerney, J.O., Mooney, J., Powell, R., Haikara, A. and Moran, A.P. (1995) Sequence of the gene encoding the 16S rRNA of the beer spoilage organism *Megasphaera cerevisiae*. *J Ind Microbiol* **15**, 67–70.

Haikara, A. (1989) Invasion of anaerobic bacteria into pitching yeast. *Proceedings of the European Brewing Convention*, 22nd edn. pp. 537–544. New York, NY: Oxford University Press.

Henriksson, E. and Haikara, A. (1991) Airborne microorganisms in the brewery filling area and their effect on microbiological stability of beer. *Monats Brauwissen* **1**, 4–8.

Juvonen, R. and Suihko, M.L. (2006) *Megasphaera paucivorans* sp. nov., *Megasphaera sueciensis* sp. nov. and *Pectinatus haikarae* sp. nov., isolated from brewery samples, and emended description of the genus *Pectinatus*. *Int J Syst Evol Microbiol* **56**, 695–702.

Juvonen, R., Koivula, T. and Haikara, A. (2008) Group-specific PCR-RFLP and real-time PCR methods for detection and tentative discrimination of strictly anaerobic beer-spoilage bacteria of the class Clostridia. *Int J Food Microbiol* **128**, 162–169.

Kingsley, V.V. and Hoeniger, J.F.M. (1973) Growth, structure and classification of *Selenomonas*. *Bacteriol Rev* **37**, 479–521.

Matoulková, D. (2008) Strictly anaerobic bacteria in beer and in breweries. *Kvasny Prum* **54**, 338–343.

Matoulková, D., Kosař, K. and Sigler, K. (2012a) Rapid, simple and specific cultivation-based method for detection of *Pectinatus* sp. in brewery samples. *J Am Soc Brew Chem* **70**, 29–34.

Matoulková, D., Kosař, K., Slabý, M. and Sigler, K. (2012b) Occurrence and species distribution of strictly anaerobic bacterium *Pectinatus* in brewery bottling halls. *J Am Soc Brew Chem* **70**, 262–267.

Motoyama, Y. and Ogata, T. (2000) 16S-23S rDNA spacer of *Pectinatus*, *Selenomonas* and *Zymophilus* reveal new phylogenetic relationships between these genera. *Int J Syst Evol Microbiol* **50**, 883–886.

Paradh, A.D., Mitchell, W.J. and Hill, A.E. (2011) Occurrence of *Pectinatus* and *Megasphaera* in the major UK breweries. *J Inst Brew* **117**, 498–506.

Ricke, S.C., Martin, S.A. and Nisbet, D.J. (1996) Ecology, metabolism, and genetics of ruminal selenomonads. *Crit Rev Microbiol* **22**, 27–56.

Satokari, R., Juvonen, R., Mallison, K., von Wright, A. and Haikara, A. (1998) Detection of beer spoilage bacteria *Megasphaera* and *Pectinatus* by PCR and colorimetric microplate hybridization. *Int J Food Microbiol* **45**, 119–127.

Schleifer, K.H., Leuteritz, M., Weiss, N., Ludwig, W., Kirchhof, G. and Sedel-Rüfer, H. (1990) Taxonomic study of anaerobic, gram-negative, rod-shaped bacteria from breweries: emended description of *Pectinatus cerevisiiphilus* and description of *Pectinatus frisingensis* sp. nov., *Selenomonas lacticifex* sp. nov., *Zymophilus rafinosivorans* gen. nov., sp. nov., and *Zymophilus paucivorans* sp. nov. *Int J Syst Bacteriol* **40**, 19–27.

Seidel-Rüfer, H. (1990) *Pectinatus* and other morphologically similar Gram-negative anaerobic bacilli from brewing. *Monatssch Brauwiss* **43**, 101–105.

Shouche, Y.S., Dighe, A.S., Dhotre, D.P., Patole, M.S. and Ranade, D.R. (2009) Genus XXI. *Selenomonas*. In *Bergey's Manual of Systematic Bacteriology*, vol 3, 2nd edn eds. De Vos, P., Garrity, G.M., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.H. and Whitman, W.B. pp. 1106–1112. New York, NY: Springer.

Storgards, E., Tapani, K., Hartwall, P., Saleva, R. and Suihko, L.M. (2006) Microbial attachment and biofilm formation in brewery bottling plants. *J Am Soc Brew Chem* **64**, 8–15.

Suzuki, K. (2011) 125th Anniversary review: microbiological instability of beer caused by spoilage bacteria. *J Inst Brew* **117**, 131–155.

Timke, M., Wolking, D., Wang-Lieu, W.Q., Altendorf, K. and Lipski, K. (2005) Microbial composition of biofilms in a brewery investigated by fatty acid analysis, fluorescence in situ hybridisation and isolation techniques. *J Appl Microbiol Biotechnol* **66**, 100–107.

Vaughan, A., O'Sullivan, T. and van Sinderen, D. (2005) Enhancing the microbiological stability of malt and beer – a review. *J Inst Brew* **111**, 355–371.

Vávrová, A., Matoulková, D., Balážová, T. and Šedo, O. (2014) MALDI-TOF MS analysis of anaerobic bacteria isolated from biofilm-covered surfaces in brewery bottling halls. *J Am Soc Brew Chem* **72**, 95–101.