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In situ detection of a virulence factor mRNA and 16S rRNA in *Listeria monocytogenes*

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Abstract

Simultaneous in situ analysis of the structure and function of bacterial cells present within complex communities is a key for improving our understanding of microbial ecology. A protocol for the in situ identification of *Listeria* spp. using fluorescently tagged, rRNA-targeted oligonucleotide probes was developed. Ethanol fixation and enzymatic pretreatment with lysozyme and proteinase K were used to optimize whole cell hybridization of exponential phase and stationary phase *Listeria* spp. cells. In parallel, transcript probes carrying multiple digoxigenin molecules were combined with anti-digoxigenin Fab antibody fragments labeled with horseradish peroxidase to detect, via the catalytic deposition of fluorescein-tyramide, the *iap*-mRNA in single *Listeria monocytogenes* cells. The *iap* gene encodes the associated virulence factor p60. Application of the new signal amplification technique resulted in strong signals comparable in intensity to those obtained with fluorescently labeled rRNA-targeted oligonucleotide probes.

Keywords: In situ hybridization; mRNA; 16S rRNA; *Listeria monocytogenes*; Virulence factor

1. Introduction

Fluorescent in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes is a powerful tool to analyze in a cultivation-independent way the structure and dynamics of complex microbial communities (e.g. [1,2]). Combination of FISH with confocal laser scanning microscopy also permits analysis of the spatial distribution of probe-target populations in microbial biofilms (e.g. [2,3]) and tissue samples [4]. While extremely useful for identification, rRNA-based techniques usually do not provide the researcher with much information about in situ functions of bacteria within their respective ecosystems. Molecular techniques for the in situ visualization of mRNA of key functional proteins in single microbial cells would provide the opportunity to narrow this gap and to link abundance and spatial distribution of bacteria with their in situ physiological activities.

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Due to the low number and instability of most mRNA molecules in prokaryotic cells only a few reports of successful in situ detection of bacterial mRNA have been reported to date [5–9].

Listeria spp. are facultatively anaerobic, non-spore-forming rod-shaped bacteria which have been assigned by ribosomal DNA sequence analysis to the Gram-positive bacteria with low DNA G+C content [10]. The genus *Listeria* comprises six well characterized species, among which *L. monocytogenes* is of particular importance as a pathogen causing serious food-borne infections in animals and humans. Newborns, pregnant women and immunocompromised individuals are at increased risk for infection with virulent strains of *L. monocytogenes*. Such strains are able to display an intracellular life cycle within nonprofessional phagocytes as well as macrophages and monocytes. Several proteins have been recognized as essential for the virulence of *L. monocytogenes* [11]. Among these, the 60-kDa extracellular p60 protein, encoded by the *iap* (invasion associated protein) gene has been identified to be not only required for efficient adhesion to certain mammalian cell types but also essential for cell metabolism of *L. monocytogenes* due to its murein hydrolase activity [12]. It has been shown that all *Listeria* species secrete a p60 protein, albeit with genus-specific conserved N- and C-termini, and species-specific variable internal portions [13].

In the present study we report on a rRNA-based in situ identification protocol for the genus *Listeria*. In addition we have developed a technique for in situ detection of *iap*-mRNA within intact cells of *L. monocytogenes*. To our knowledge this is the first time that successful in situ identification of mRNA from a virulence factor in bacteria has been demonstrated. This technique might offer the opportunity to analyze gene expression both in *L. monocytogenes* present in mixed microbial communities and during infection in mammalian cells.

2. Materials and methods

2.1. Bacterial strains and media

Five *L. monocytogenes* strains (sv4a L99, sv4b SLCC 4013, sv1/2a EGD PrfA$^-$, sv1/2a EGD ActA$^-$, RIII) as well as *L. seeligeri* SLCC 3954, *L. grayi*, *L. welshimeri* SLCC 5828, *L. innocua* sv6a NCTC 11288, and *L. ivanovii* ATCC 19119 were obtained from the Institute of Medical Microbiology and Hygiene (University of Würzburg, Würzburg, Germany). *Listeria* strains and *Enterococcus hirae* DSM 20160^T were cultured aerobically in brain heart infusion (BHI) broth at 37°C. *Listeria* cells were harvested at various growth phases (see Section 3) by centrifugation (5000×*g* for 5 min). *Escherichia coli* ATCC 11775^T, *Pseudomonas aeruginosa* DSM 2659, and *Aeromonas hydrophila* ATCC 7966^T were grown aerobically in Luria-Bertani medium and harvested at mid-exponential phase by centrifugation (5000×*g* for 5 min) to obtain cells with high rRNA contents.

2.2. Oligonucleotide probes

The following rRNA-targeted oligonucleotide probes were used: (i) LIS-1255, complementary to a region of the 16S rRNA of the genera *Listeria* and *Brochothrix* [14]; (ii) GAM, complementary to a region of the 23S rRNA conserved in the gamma subclass of Proteobacteria [15]; (iii) EUB, complementary to a conserved region of bacterial 16S rRNA molecules [16]. In addition four oligonucleotides were designed and used for detection of *iap*-mRNA: (i) iap-30 (5′-ACCGCAATCCCAGCTG-TA-3′, derived from primer UnilisA [13]); (ii) iap-630 (5′-CCCAAATAGTGTCACCGC-3′), (iii) iap-1159 (5′-AGTTGGTCCGTTACCACC-3′); and (iv) iap-1437 (5′-TACGCGACCGAAGCCA-3′, a truncated version of primer iap-1436; see below). Oligonucleotides were synthesized with a C6-TFA aminolinker at the 5′ end (Interaktiva, Ulm, Germany). Labeling with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (CFLUOS; Boehringer, Mannheim, Germany), the monofunctional, hydrophilic sulfoindocyanine dyes Cy3 and Cy5 (Amersham, Buckinghamshire, UK) or horseradish peroxidase (HRP) was performed as described previously [17,18].

2.3. Cell fixation and enzymatic pretreatment

Gram-negative reference cells were fixed by addition of paraformaldehyde solution (final concentra-

tion 4%) and stored in a 1:1 mixture of phosphate-buffered saline (130 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2) and 96% ethanol at −20°C [17]. *Listeria* cells were fixed either by the procedure used for Gram-negative cells or by addition of ethanol to a final concentration of 50% (v/v) [19]. *E. hirae* cells were fixed by addition of ethanol [19]. *Listeria* cells were spotted on precleaned six-well glass slides, dried at 46°C and dehydrated in 50, 80, and 96% ethanol (3 min each). For partial enzymatic digestion of the cell wall *Listeria* cells were treated with and without lysozyme and proteinase K prior to hybridization. In the developed optimized protocol fixed immobilized *Listeria* cells were covered with 20 μl of lysozyme solution (10 mg ml^{-1} of lysozyme [Serva, Heidelberg, Germany; 164.500 U mg^{-1}] in a buffer containing 100 mM Tris-HCl and 50 mM EDTA, pH 8.0) and incubated for 20 min at room temperature. Lysozyme reaction was stopped by rinsing the slide thoroughly with dH_2O followed by an additional ethanol series. Subsequently, *Listeria* cells were subjected to an aqueous proteinase K solution (0.05 mg ml^{-1}, 20 U mg^{-1}, Boehringer) and incubated for 5 min at room temperature. Finally, proteinase K was removed by rinsing with dH_2O and an additional ethanol series. For preparing artificial mixtures of Gram-negative reference cells and *Listeria* cells, Gram-negative reference cells were spotted on the well containing the fixed *Listeria* cells after the enzymatic cell wall permeabilization procedure.

2.4. Whole cell hybridization with oligonucleotide probes

Hybridizations with CFLUOS, Cy3, Cy5 and HRP-labeled oligonucleotide probes were performed following the protocols of Manz et al. [15] and Amann et al. [18]. Specific hybridization with probe Lis-1255 required the addition of formamide to a final concentration of 35% to the standard hybridization buffer. Hybridizations with the *iap*-mRNA-targeting oligonucleotide probes were performed by simultaneous application of the four probes (each labeled with Cy3) using a range of formamide concentrations (0%, 10%, 20%, 30%, 40%, 50%, 60%) in the hybridization buffer. The NaCl concentration in the washing buffer was adjusted according to the formamide concentration in the respective hybridization buffer [15]. Specifically bound horseradish peroxidase-labeled oligonucleotide probes were detected by addition of the substrate fluorescein tyramide as described by Schönhuber et al. [20]. For dual staining of *L. monocytogenes* cells with fluorescent oligonucleotide probes and DAPI, cells were stained after in situ hybridization with 1 μg ml^{-1} DAPI for 5 min at RT. Fluorescence was detected with a Zeiss LSM 510 scanning confocal laser microscope (Carl Zeiss, Oberkochen, Germany).

2.5. DNA isolation, PCR amplification, cloning and sequencing

High molecular mass genomic DNA was extracted from *L. monocytogenes* sv1/2a EGD $ActA^-$ using a modification of the protocol given by Lema et al. [21]. For cell lysis we replaced the lysozyme step by an ultrasonic treatment (150 s) after the addition of an equal volume of glass beads (0.1–0.11 mm diameter; Braun, Melsungen, Germany). PCR primers iap-1159F (5′-GGTGGTAACGGACCAACT-3′) and iap-1436R (5′-ATACGCGACCGAAGCCAA-3′; derived from the primer Lis1B [13]) were used for PCR with a thermal capillary cycler (Idaho Technology, Idaho Falls, ID). The primer pair bound between nucleotide position 1159 and 1453 of the *iap* gene (nucleotide numbering in *L. monocytogenes* EGD [13]). Reaction mixtures were prepared according to the instruction of the manufacturer in a total volume of 50 μl by using the 20 mM $MgCl_2$ reaction buffer. Thermal cycling was carried out as follows: an initial denaturation at 94°C for 45 s followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 58°C for 25 s and elongation at 72°C for 22 s. Cycling was completed by a final elongation step at 72°C for 60 s. The presence and size of the amplification product were determined by agarose gel electrophoresis (1.0%). The amplified 294 bp long product was excised from the gel and purified using the Gel DNA Extraction Kit (Boehringer). Purified products were ligated into the cloning vector pCR2.10 and transformed into competent *E. coli* INVαF′ cells with the Original TA cloning kit (Invitrogen Corp., San Diego, CA). Identity and orientation of the inserts were determined by cycle sequencing of purified plasmid preparations (QIAgen Spin Plasmid Kit; Qiagen, Hilden, Germany) with a

Thermo sequenase cycle sequencing kit (Amersham) and an infrared automated DNA sequencer (LiCor, Inc., Lincoln, NE) under the conditions recommended by the manufacturers.

2.6. In vitro transcription

Digoxigenin (DIG)-labeled antisense and sense polyribonucleotide probes were generated by in vitro transcription from pCR2.10 vectors containing the 294 bp long, cloned *iap* fragments in the respective orientations. 396-nucleotide sense and antisense transcripts were transcribed from the T7 promoter after linearization of the plasmid with *Spe*I. Transcription reactions were performed with an RNA transcription kit (Boehringer) with a UTP/DIG-11-UTP ratio of 6.5:3.5. Transcription products were analyzed by denaturing polyacrylamide gel electrophoresis and spectrophotometrically following standard protocols [22].

2.7. Northern blot analysis

Total RNA was isolated from *L. monocytogenes* strains sv1/2a EGD ActA$^-$ and 7972 PrfA$^-$, as well as from *P. aeruginosa*, as described by Oelmüller et al. [23]. For Northern blotting, formaldehyde-agarose gel electrophoresis was performed as described previously [22]. DIG-labeled RNA molecular mass markers (Boehringer) were also loaded onto the gel for subsequent use as standards for the size determination of hybridizing RNA bands. After electrophoresis, nucleic acids were transferred with a pressure blotter onto positively charged nylon membranes (Hybond™-N$^+$, Ver. 2,) and subsequently crosslinked by exposure to UV light for 150 s. Hybridizations were performed with HRP-labeled oligonucleotides or with DIG-conjugated polyribonucleotide probes. The membranes were prehybridized for 1 h at 46°C (polyribonucleotide probes) or at 35°C (oligonucleotide probes) with 20 ml solution containing 10.8×SSC, 4.3% blocking reagent (Boehringer), 0.2% *N*-laurylsarcosine and 0.04% SDS. Hybridizations were performed at 46°C for at least 12 h using polyribonucleotide probes. Oligonucleotide hybridizations were performed at 35°C for 90 min. Hybridization solution contained 0.9 M NaCl, 0.02 M Tris-HCl (pH 8.0), 1.8% blocking reagent, 0.1% *N*-lauryl-sarcosine, 0.01% SDS, 22% formamide (EUB, *iap*-mRNA-targeted oligonucleotide probes) or 40% formamide (polyribonucleotide probes) or 62% formamide (Lis-1255), and 100 ng of HRP-labeled oligonucleotide probe or 250 ng DIG-labeled polyribonucleotide probe. Following hybridization, membranes were washed twice at 35°C (oligonucleotide probes) or 48°C (polyribonucleotide probes) for 10 min (oligonucleotide probes) or 15 min (polyribonucleotide probes) in 10 ml washing buffer containing 20 mM Tris-HCl (pH 7.2), 4.6 mM EDTA, and 0.01% SDS. NaCl concentration in the washing buffer was adjusted according to the formamide concentration in the respective hybridization buffer [15]. DIG-conjugated polyribonucleotide probes were detected with HRP-labeled anti-DIG Fab antibody fragments following instructions recommended by the manufacturer (Boehringer). Specifically bound nucleic acid probes were visualized via HRP-induced chemiluminescence (ECL; Amersham). Emitted light was recorded with an ultrasensitive photon-sensitive camera (Hamamatsu C2400-47, Hamamatsu Photonics, Hersching, Germany).

2.8. Whole cell hybridization with DIG-conjugated polyribonucleotide probes

Fixed and enzymatically pretreated cells were covered with 10 μl hybridization mixture (75 mM NaCl, 80% formamide, 20 mM Tris, 0.01% SDS, 250 ng of transcript probe) in an isotonically equilibrated humid chamber. Following an initial denaturation at 80°C for 30 min, hybridization was performed at 46°C for 5 h. Hybridization was terminated by immersing the slides briefly in distilled H_2O. After air-drying, wells were covered with 21 μl detection solution (20 mM Tris, 0.7 M NaCl, 1% blocking reagent) containing 0.6 μg HRP-labeled anti-DIG antibody Fab fragments. Slides were incubated at 27°C for 1 h, washed for 10 min at the same temperature in a solution containing 0.7 M NaCl and 100 mM Tris, and finally rinsed with distilled water. Specifically bound HRP-labeled Fab fragments were detected by using the substrate fluorescein tyramide [20]. Subsequently, fixed cells were hybridized with Cy5-labeled oligonucleotide probes (see above). Fluorescence was observed with a Zeiss LSM 510 scanning confocal laser microscope (Carl Zeiss).

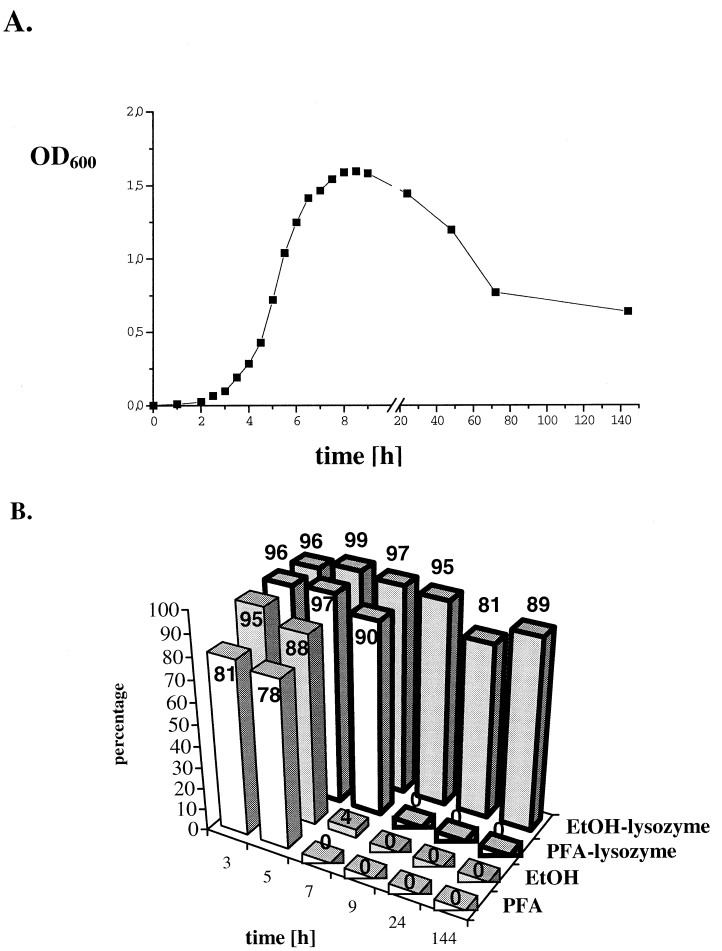


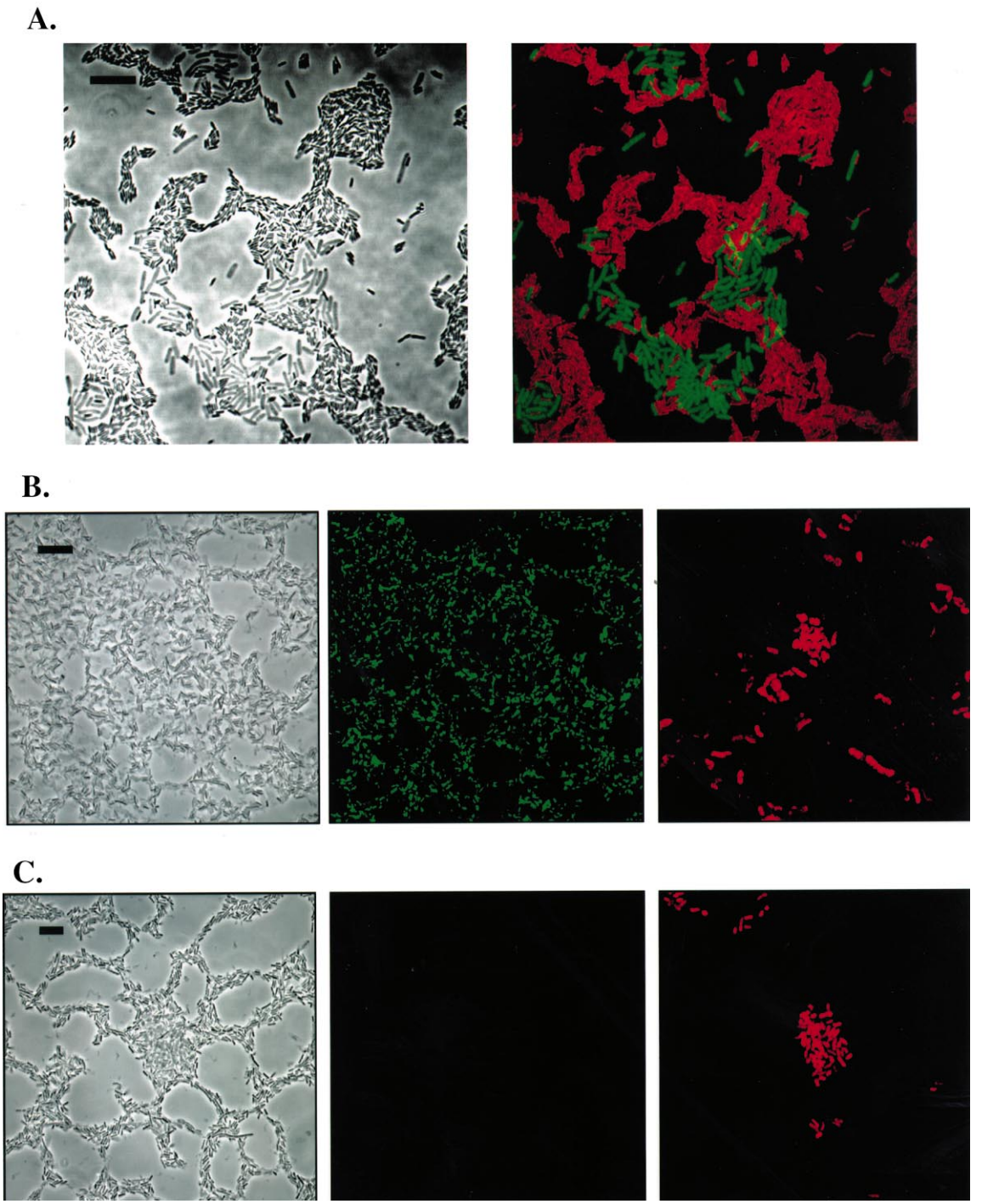
Fig. 1. The effect of fixative and lysozyme treatment on detection efficiency of *L. monocytogenes* by whole cell hybridization. A: Growth curve of *L. monocytogenes* sv1/2a EGD ActA$^-$ in BHI medium at 37°C. B: *L. monocytogenes* sv1/2a EGD ActA$^-$ cells were fixed at various time points of the growth curve (3, 5, 7, 9, 24, and 144 h) with ethanol (EtOH) or paraformaldehyde (PFA) and optionally treated with lysozyme prior to whole cell hybridization with Cy3-labelled probe Lis-1255. The Lis-1255/DAPI ratio was determined following staining with DAPI.

3. Results and discussion

3.1. *In situ hybridization of Listeria spp. with rRNA-targeted oligonucleotide probes*

The difference in cell wall composition and structure between Gram-negative and Gram-positive bacteria hampers the application of in situ hybridization techniques established for Gram-negative bacteria to Gram-positive bacteria. Previous work has demonstrated that successful in situ hybridization of most Gram-positive bacteria requires changes in the fixation protocol and/or additional enzymatic pretreatments to make them permeable for reporter molecule-labeled nucleic acid probes (e.g. [19,24,25]). Therefore, we initially tested different fixation and enzymatic pretreatments to adapt existing in situ hybridization protocols to *Listeria* spp.

BHI-cultivated *L. monocytogenes* sv1/2a EGD ActA$^-$ cells were harvested at different times during the growth curve (Fig. 1A) and fixed immediately by addition of either paraformaldehyde or ethanol. Subsequent in situ hybridization with the 16S rRNA-targeted oligonucleotide probe Lis-1255 revealed



that permeabilization of *Listeria* cells due to ethanol fixation was more effective than with paraformaldehyde fixation (Fig. 1B). Both fixatives allowed the specific visualization of the majority of exponential

Fig. 2. In situ detection of 16S rRNA and *iap*-mRNA in *L. monocytogenes*. A: Artificial mixture of *E. coli* and stationary phase *L. monocytogenes* sv1/2a EGD ActA$^-$ (fixed after 9 h growth in BHI medium) simultaneously hybridized with CFLUOS-labeled probe GAM (green) and Cy3-labeled probe Lis-1255 (red). B, C: Artificial mixture of *L. monocytogenes* sv4b and *Aeromonas hydrophila* hybridized with multiply DIG-labeled *iap*-mRNA antisense polyribonucleotide probe (B, center) or with multiply DIG-labeled *iap*-mRNA sense polyribonucleotide probe (C, center). Intracellular, probe-conferred DIG molecules were detected via HRP-labeled anti-DIG Fab fragments and fluorescein tyramide (green). For both experiments *A. hydrophila* cells were visualized by a subsequent hybridization with Cy5 monolabeled oligonucleotide probe GAM (red; B and C, right). During image acquisition with the confocal laser scanning microscope all settings (laser intensity, pinhole size, contrast and brightness, scanning time, and number of averages) were identical for panels B and C. Phase-contrast (left) and epifluorescence (center, right) micrographs are shown for identical microscopic fields. Bars represents 10 μm. ←

Listeria cells while stationary cells were not detectable. Failure to detect stationary *Listeria* cells could be due to low cellular ribosome contents or to limited cell wall permeability. We determined the effect of treating ethanol- and paraformaldehyde-fixed *Listeria* cells with different concentrations of lysozyme (0.1–50 mg ml^{-1}) on probe permeability. These experiments demonstrated that BHI-cultivated stationary *Listeria* cells (i) still possess sufficient ribosomes for in situ detection (Fig. 1B) and (ii) are permeable for fluorescently labeled oligonucleotide probes after being treated with 10 mg ml^{-1} lysozyme for 20 min at RT (Fig. 2A). Increasing lysozyme concentration or exposure time resulted in partial or complete lysis of the cells. At a formamide concentration of 35% (v/v), probe Lis-1255 hybridized strongly with representative members of all six *Listeria* species and not with non-target organisms (Table 1). Fluorescent in situ hybridization is a promising tool for rapid identification and quantification of *Listeria* spp. in complex samples and could become a valuable complement to standard cultivation, PCR and immunological techniques. However, *Listeria* cells present in natural samples might differ in their cell wall composition from BHI-cultivated cells. Consequently, it is recommended to perform in situ monitoring of *Listeria* in samples which have been subjected to different cell permeabilization procedures.

3.2. *In situ detection of mRNA of the iap gene in whole fixed cells of L. monocytogenes*

The *iap*-mRNA was chosen as target for the development of a nucleic acid probe-based method for monitoring specific gene expression in *L. monocytogenes* for two reasons: first, *iap*-mRNA has been reported to be regulated at a post-transcriptional level and is present in a relatively high copy number [26]; second, expression of *iap*, which encodes a murein hydrolase involved in septum separation, is essen-

Table 1
Listing of strains analyzed by whole cell hybridization with probe Lis-1255

Organism	Source[a]	Lis-1255	EUB
Listeria monocytogenes sv1/2a EGD ActA$^-$	BW	+	+
Listeria monocytogenes sv1/2a EGD PrfA$^-$	BW	+	+
Listeria monocytogenes RIII	SLCC 5779	+	+
Listeria monocytogenes sv4a L99	BW	+	+
Listeria monocytogenes sv4b	SLCC 4013	+	+
Listeria grayi	BW	+	+
Listeria innocua sv6a	NCTC 11288	+	+
Listeria ivanovii	ATCC 19119	+	+
Listeria seeligeri	SLCC 3954	+	+
Listeria welshimeri	SLCC 5828	+	+
Escherichia coli	ATCC 11775^T	−	+
Enterococcus hirae	DSM 20160^T	−	+

[a]BW, Biozentrum Universität Würzburg, Würzburg, Germany; ATTC, American Type Culture Collection, Rockville, MD, USA; NCTC, National Collection of Type Cultures, London, UK; SLCC, Special *Listeria* Culture Collection, Institut für medizinische Mikrobiologie und Hygiene, Universität Würzburg, Würzburg, Germany; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

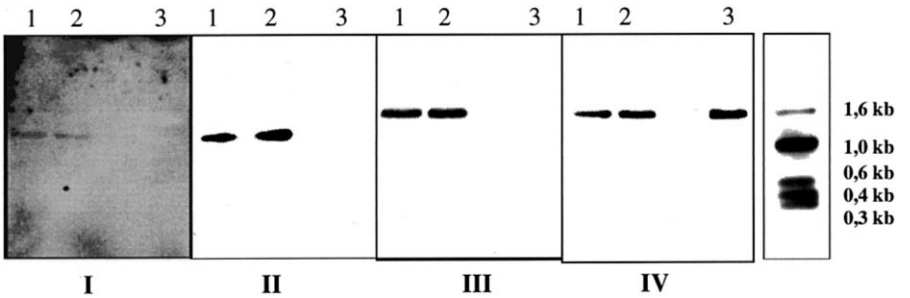


Fig. 3. Northern blot detection of *iap*-mRNA and 16S rRNA in *L. monocytogenes*. Membranes were hybridized with: (I) four *iap*-mRNA targeting HRP-labeled oligonucleotide probes (*iap*-mRNA detection with multiple oligonucleotide probes required prolonged exposure times resulting in higher membrane background); (II) a multiply DIG-labeled *iap*-mRNA antisense polyribonucleotide probe; (III) HRP-labeled 16S rRNA-directed probe Lis-1255; (IV) HRP-labeled 16S rRNA-targeting probe EUB. Lane 1, *L. monocytogenes* sv1/2a EGD ActA$^-$ RNA; lane 2, *L. monocytogenes* sv1/2a EGD PrfA$^-$ RNA; lane 3, *P. aeruginosa* RNA.

tial for cell viability of *L. monocytogenes* [12]. Northern analysis using direct photon camera inspection demonstrated that *iap*-mRNA concentrations in *L. monocytogenes* were similar in cells from exponential and stationary growth phases (data not shown). However, there is less *iap*-mRNA than 16S rRNA in the cells (Fig. 3). Low numbers of *iap*-mRNA required the simultaneous application of the four *iap*-mRNA-targeted oligonucleotide probes to obtain a detectable signal after Northern hybridization (Fig. 3I). The sensitivity of the *iap*-mRNA Northern hybridization was significantly increased by using multiply labeled antisense polyribonucleotide transcript probes (Fig. 3II). Polyribonucleotide-conferred *iap*-mRNA-specific Northern hybridization signals were comparable in their intensity to those obtained with 16S rRNA-targeted oligonucleotide probes labeled with a single HRP molecule (Fig. 3III, IV). Specificity of polyribonucleotide hybridization was demonstrated by hybridization of the Northern blot membranes with a multiply labeled sense probe which did not lead to a detectable signal (data not shown).

Detection of *iap*-mRNA in fixed and lysozyme-treated *L. monocytogenes* cells was unsuccessful using the four *iap*-mRNA-targeted Cy3-labeled oligonucleotide probes simultaneously for in situ hybridization (data not shown). Increase of the formamide concentration in the hybridization buffer in order to remove higher order structure in the target mRNA did not result in detectable signals. The most probable reason for this was the relatively low cellular copy number of the *iap*-mRNA (see Section 2.7) which could not even be sufficiently compensated for by the simultaneous use of four oligonucleotide probes labeled with the currently brightest fluorescent dye Cy3 [27]. In order to further increase the sensitivity of our assay we attempted to use HRP-labeled derivatives of the four *iap*-mRNA-directed oligonucleotide probes in combination with the fluorogenic TSA assay. This approach can confer up to 20-fold more fluorescence to target cells than can oligonucleotides labeled with a single fluorescent dye molecule [18]. It requires, however, that the target cells are permeable for the relatively large oligonucleotide-enzyme conjugate. Hybridization of ethanol-fixed and lysozyme-pretreated *L. monocytogenes* cells with a HRP-labeled derivative of oligonucleotide probe Lis-1255 revealed that less than 10% of the cells could be visualized via enzymatic transformation of fluorescein tyramide. To further enhance cell permeability we implemented an additional optimized proteinase K treatment (0.05 mg ml^{-1}; 5 min, RT) in the hybridization protocol. This resulted in specific visualization of more than 90% of the *L. monocytogenes* cells with HRP-labeled probe Lis-1255 (data not shown). However, heterogeneity in signal intensities between different *L. monocytogenes* cells, indicating differences in cell wall composition, was observable. This effect was most pronounced for the isogenic mutants *L. monocytogenes* sv1/2a EGD ActA$^-$ and PrfA$^-$, and less pronounced for *L. mono-*

cytogenes sv4b which was therefore selected as a model strain for subsequent studies. Simultaneous hybridization of ethanol-fixed, lysozyme- and proteinase K-pretreated *L. monocytogenes* sv4b with HRP-labeled derivatives of the four *iap*-mRNA-targeted oligonucleotide probes, however, still did not lead to detectable signals using the fluorogenic TSA system (data not shown), indicating that the cellular concentration of *iap*-mRNA is below the detection limit of the HRP-oligonucleotide-TSA technique.

For further sensitivity enhancement we applied a multiply DIG-labeled *iap*-mRNA antisense polyribonucleotide probe. Such polyribonucleotide probes have been recently shown to be nicely suited for signal amplification in in situ hybridization techniques [28] and have therefore been applied for non-fluorogenic detection of bacterial mRNA [5,7]. After detection of probe-conferred DIG via a HRP-labeled anti-DIG Fab antibody fragment and the fluorogenic TSA system, *L. monocytogenes* sv4b cells showed bright signals while *Aeromonas hydrophila* reference cells remained unstained (Fig. 2B). These results demonstrate that the developed fixation and pretreatment protocol renders *L. monocytogenes* sv4b permeable for the large, 240-kDa HRP-Fab molecules. The specificity of the signals was corroborated by control hybridizations with a sense polyribonucleotide probe (using identical conditions and reaction reagents) which did not confer detectable fluorescence to the *Listeria* cells (Fig. 2C).

In the present study we have developed a protocol for 16S rRNA-based in situ identification of bacteria of the genus *Listeria*. Moreover, we have demonstrated the usefulness of multiply labeled polyribonucleotide probes in combination with the fluorogenic TSA system for in situ monitoring of mRNA of a virulence factor in *L. monocytogenes*. Our results, however, demonstrate that successful application of the PolyDIG-transcript–HRP-Fab–TSA system requires target strain-specific optimization of the permeabilization procedure and is therefore recommended for well defined target species. The method should be especially powerful when combined with rRNA hybridization techniques and confocal microscopy to study activities of pathogenic bacteria during infection or of indicator species in complex environments.

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