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Full Length Research Paper

Identification of a Beta-Glucosidase in Listeria Monocytogenes EGD and Characterization of its Gene Product

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ABSTRACT

The bglA-gene, encoding a b-glucosidase from *Listeria monocytogenes* has been cloned by complementation of an *Escherichia coli bglB* mutant. The primary sequence of bglA comprised 471 amino acids and showed strong overall homology to known bacterial glycosyl hydrolases. The *bglA* gene was preceded by a highly conserved binding motif for a ribonucleic antiterminator (RAT) which overlapped a rho-independent terminator sequence. A plasmid harboring the *bglA* gene expressed a unique polypeptide of 54 kDa as determined by maxi-cell analysis which was in excellent correspondence with its predicted molecular weight. Purified recombinant BglA exhibited enzymatic activity on o-nitrophenyl-b-D-galactopyranoside (ONPG) with a Michaelis constant K_M of 9.71 x 10^{-4} M for this substrate. Using RT-PCR low levels of transcription of the *bglA*-gene in *L. monocytogenes* was detected in Luria-Bertani (LB) broth cultures growing in LB broth alone. An isogenic *bglA*-deficient mutant was not deficient for glucosidase production indicating the presence of other genes with b-glucosidase activity in *L. monocytogenes*. PCR analysis revealed that *bglA* gene was present in the non-pathogenic strains *L. seeligeri*, *L. innocua* , and *L. welshemeri* as well but absent in *L. grayi* and in the animal pathogen *L. ivanovii*. In concordance with data presented above, low stringency hybridizations with a *bglA* specific probe indicated the presence of additional *bglA*-like sequences in the *L. monocytogenes* genomes.

Key words: *Listeria monocytogenes,* beta-glucosidase, protein purification

1. INTRODUCTION

Members of the genus *Listeria* are gram-positive ubiquitous living bacteria isolated from soil, water, and decaying plants. Their primary habitat is decaying vegetation where these bacteria grow saprophytically. DNA-DNA hybridization studies, 16S rRNA sequencing and multilocus enzyme analysis demonstrate that this genus comprises two lines of descent: one comprises *L. monocytogenes* and the genomically closely related species *L. innocua*, *L. ivanovii*, *L. welshimeri*, and *L. seeligeri* while the other contains a single species, *L. grayi*. *L. monocytogenes* is known to be a human and animal pathogen whereas *L. ivanovii* is only pathogenic for animals. All of the other members of these species are non-pathogenic (Waminathan, et al. 1995). *L. monocytogenes*, a facultative intracellular bacterium, is the causative agent of listeriosis. Infection can occur in predisposed persons such as the elderly, the immunocompromised or the unborn and is usually preceded by consumption

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of *Listeria*-contaminated foodstuffs such as soft cheese or unpasteurized milk (Farber and Peterkin 1991). The infectious process of *L. monocytogenes* can be separated into different steps including adhesion, invasion, escape from a phagolysomal compartment, intracytosolic replication, actin-based intracellular motility and cell-to-cell spread. Many of the genes required for intracellular survival and growth have been identified and found to be clustered on the listerial chromosomee (Chakraborty and Wehland 1997; Ireton and Cossart 1997). These include the internalins (InlA, InlB), lysteriolysin (Hly), two phospholipases (PlaC, PlcB) with differing specificities, a metalloprotease (Mpl) ant the actin nucleating factor ActA. All of these virulence factors are coordinately regulated by the transcriptional activator protein PrfA (Portnoy et al. 1992).

The ability of *Listeria* in food and environmental samples to the hydrolysis of the plant β-D-glucoside esculin (6,7-dihydroxy-coumarin-β-D-glucoside) has become a widely-used tool in the differenciation of this species. This sugar is hydrolyzable by all *Listeria* species to β-D-glucopyranose and 6,7-dihydroxycoumarin (esculetin) and in the presence of Fe^{3+} a black halo of a esculetin-Fe^{3+}-complex is detectable around bacterial colonies exhibiting β-D-glucosidase activity (Cowart and Foster 1985; Curtis et al. 1989; Edberg et al. 1977; Fraser and Sperber 1988; James et al. 1997; Siragusa et al. 1990; Trepeta and Edberg 1987). Carbonhydrates also play important roles for bacteria in survival in the environment in adaptive responses. The various sugar phosphotransferase systems and regulated osmoregulative processes contribute to optimized growth of the bacteria in different ecological niches (Reizer 1989; Saier 1989; Stock et al. 1989).

Recently, evidence has been accruing to implicate the role of different sugars in the regulation of virulence factors in *L. monocytogenes*. Thus, both the expression of listeriolysin (*hly*) and the phosphatidylinositol-specific phospholipase C (*plcA*) was shown to be repressed by cellobiose whereas arbutin, a phenolic β-Dglucoside was shown to regulate listeriolysin expression alone (Park 1994; Park and Kroll 1993). It has also been demonstrated that the utilization of glucose-1-phosphate was PrfA-dependent and coordinately expressed with virulence factors in this pathogen (Ripioet al. 1997). Nevertheless, data by Milenbachs and colleagues indicate that the concentrations of cellubiose and other sugars used as supplements in the culture medium significantly enhanced growth of *L. monocytogenes*, suggesting that the repression phenomenon probably results from a metabolic effect of sugar utilization rather than a signal-sensing response (Milenbachs et al. 1997).

In this study we have identified a b-glucosidase from *L. monocytogenes* with significant overall sequence homology to members of the family of glycosyl hydrolases. We present evidence for the genetic organization of the isolated gene, designated *bglA*, enzymatic activity of purified recombinant BglA, and its expression under various conditions of growth.

2. MATERIALS & METHODS

2.1 Bacterial strains, media, and reagents

The weakly hemolytic *Listeria monocytogenes* strain EGD serotype 1/2a was originally obtained from G. B. Mackaness and described previously (Kaufmann 1984). The strain served as

the parental strain for cloning of *bglA* and construction of the isogenic ΔbglA2 deletion mutant. The listerial strains *L. innocua*, *L. ivanovii*, *L. welshimeri*, and *L. grayi* were obtained from Seeligers Listeria Culture Collection (SLCC ; Institute for Medical Microbiology and Hygiene , Mannheim, Germany). Listeria strains were grown in brain heart infusion broth (BHI, DIFCO) at either 28°C, 37°C, or 42°C and 5 μg of erythromycin per ml was added as it was appropriate.

E. coli strain INVαF' [end, rec, hsdR17(r^{-k}, m^{+k}), supE44, λ–, thi-1, gyrA, relA1, ϕ80, lacZαΔ(lacZY-argF), deoR+, F-] (Invitrogen, Netherlands) was used for cloning and transformation. It was cultured in Luria-Bertani (LB) and supplemented either with 100 μg ampicillin per ml for multiplication of pCRII plasmids (Invitrogen) or with 300 μg erythromycin per ml multiplication of suicid plasmid pAUL-A (Chakraborty et al. 1992) and its derivates. Complementation analysis of an *E. coli* bglB mutation was performed using strain MA 223 and was indicated by colony color on Mac Conkey arbutin plates (Mahadevan et al. 1987a).

Maxicell analysis of plasmid-encoded polypeptides was performed with *E. coli* CSH26ΔF6 strain [ara thi Δ(lac-pro)Δ(recA-srlF6)rpsL] (Domann et al. 1991).

Restriction analysis and plasmid constructions were done by standard techniques as outlined by Sambrook at al. (Sambrook et al. 1989). Chemical reagents were purchased from SGMA (Deisenhofen, Germany) and MERCK (Darmstadt, Germany), unless indicated otherwise.

2.2 Determination of nucleic acid sequence

The DNA sequence of the cloned *L. monocytogenes* DNA was determined from double-stranded plasmid templates by dideoxy-chain termination (Sanger et al. 1977). Double-stranded templates were denatured, and the sequencing reactions were carried out with T7 DNA polymerase as suggested in the "Plsmid FdATP+AutoreadKit" from Pharmacia (Freiburg, Germany). Sequencing reactions were primed from vector- and custom-made oligonucleotide primers labeled at their 5' end with the fluorescent carbocyamine dye Cy5 from Pharmacia. The labeled reaction mixtures were separated by electrophoresis on 6% Hydrolink Long Ranger gels and sequences were automatically detected by a red helium neon laser (633 nm) and fixed photodiodes in the ALFexpress DNA sequencer and analyzed in the fragment analysis system from Pharmacia as outlined by the vendor.

2.3 Homology studies

To search for homologies of BglA with polypeptides published in different data bases we used the BLASTP (Altschul et al. 1990) and FASTA (Pearson et al. 1988) programs within the Heidelbeg Unix Sequence Analysis Resources-Genetics Computer Group 5HUSAR-GCG) environment at the Deutsche Krebsforschungs Zentrum, Heidelberg, Germany. Additionally, for comparing the primary peptide sequences of β-glucosidases derived from several microorganisms with the *bglA* gene product CLUSTAL V (30° and BESTFIT (Rechid et al. 1989) were used. The phylogenic tree was generated with the sequence analysis software Lasergene-MEGALIGN (DNASTAR Inc., Madison, U.S.A.).

2.4 PCR amplification

Specific DNA fragments from bacterial strains were amplified by the polymerase chain reaction (PCR) (Mullis and Faloona 1987). The specific synthesis of

the *bglA* genes from *Listeria* strains had been run on the GeneAmp PCR 2400 from Perkin Elmer (Langen, Germany) and had been carried out with the AmpliTaq® DNA polymerase under conditions described previously (Innis et al. 1990). A typical amplification profile started with an initial denaturation step at 94°C for 20 seconds, hybridization of the specific oligonucleotides to the denatured template at 55°C for 30 seconds, and extension of the annealed primers at 72°C for 90 seconds. This cycle was repeated 25 fold and the amplification profile was completed with a final extension step at 72°C for 5 minutes. In order to fuse the *bglA* gene with glutathione S-transferase 5GST° for purification of the gene product, a specific *bglA* DNA fragment was amplified by PCR using the ULTma® DNA polymerase (Perkin Elmer) with a proof reading activity and plasmid pAUL-44 as template under conditions described above. The corresponding PCR product was cloned into the expression vector pGEX-6P-1 of the GST Gene Fusion System (Pharmacia, Freiburg, Germany).

2.5 Southern hybridization

Listerial chromosomal DNA was isolated after the lysis of the bacteria (Schäferkordt et al. 1998). A total of 10 µg of the DNA was digested with either *Eco*RV, *Hind*III, *Mun*I, or *Xba*I restriction endonucleases. The digested DNA was electrophoresed on a 0.7% agarose gel for approximately 16 h (30V), after which the DNA was transferred to nylon membrane sheets (QIAbrane; QIAGEN, Hilden, Germany) as described by southern (Southern 1975). Hybridization was carried out under conditions of low stringency as described previously (Leimeister-Wächter and Chakraborty 1989). DNA probes were labeled with [α-^{32}P]dATP by the random priming technique of Freiberg and Vogelstein (Feinberg and Vogelstein 1983).

2.5 RNA isolation and reverse transcription (RT) PCR

For detection of a *bglA*-specific transcript bacteria were cultivated in Luria Bertani (LB) broth supplemented with 0.5% of arbutin, or salicin and with 1% of cellobiose, galactose, glucose, mannoside, rhamnose, sucrose or xylose (Sigma). Bacterial RNA was isolated from growing cultures (optical density at A_{600} of 1.0) of *L. monocytogenes* by the hot phenol extraction method (Leimeister-Wächter et al. 1990). For detection of a *bglA*-specific transcript during infection of host cells infected tissue culture cell lines were harvested and total RNA from eukaryotes and bacteria was isolated as described above. Copy DNA (cDNA) was generated by using the SuperScript™ preamplification system for first strand cDNA (Life Technologies, Eggenstein, Germany). The PCR for amplification of *bglA*-specific sequences was done with oligonucleotides [A] 5'-CTTCCATCGCTTGGTCCCGTATC-3' and [B] 5'-TCAAGTCAA CGCCATCTTTAATCG-3'. As control we amplified listeriolysin-specific DNA sequences with oligonucleotides [C] 5'-GCAGTTGCAAGCGCTTGGAGTGAA TGC-3' and [D]-5'CTATATTTCGGATAAAGC GTGGTGCCCC-3'.

2.6 Construction of the chromosomal in-frame deletion mutation *bglA*2

An in-frame deletion mutation in the *bglA* gene was generated as described (Chakraborty et al. 1995, Schäferkordt et al. 1998). The truncated Δ*bglA*2 polypeptide lacked the amino acid residues 31 to 436 amino acid residues or 86% of the entire gene (Fig. 1). The gene

deletion was confirmed by PCR sequencing of chromosomal DNA from Δ*bglA*2 mutant strain and Southern hybridization (data not shown).

2.7 Cloning and purification of the recombinant β-glucosidase in E. coli

A DNA fragment, encoding the mature β-glucosidase and lacking the predicted N-terminal signal peptide of 19 amino acid residues (Fig. 1), was specifically amplified by PCR employing the oligonucleotides GST/*bglA*-*Bam*HI 5'-GCTGCTGCTGGATCCCAATTCGA AGGCGCT-3' and GST/*bglA*-*Mun*I 5'-TCAAAAGCCAATTGAGAGCCAGTG AG-3'. The PCR product was digested with *Bam*HI and *Mun*I restriction endonucleases and cloned into the *Bam*HI and *Eco*RI restriction endonuclease sites of vector pGEX-6P-1, allowing direct N-Terminal fusion of gluthatione S-Transferase and a PreScission™ protease cleavage site (human rhinovirus 3C protease) to the N-terminus of BglA.

In order to purify the β-glucosidase, this plasmid pGST-*bglA* was transformed into *E. coli* strain BL21 5AGS, Heidelberg, Germany). 100 ml of LB broth (100 µg/ml ampicillin) was inoculated with the recombinant strain and was cultivated at 37°C under vigorous shaking overnight. One liter of LB broth (100 µg/ml ampicillin) ina 2 liter Erlenmeyer flask was inoculated with the overnight culture and incubated at 37°C under vigorous shaking, until the optical density A_{600} reached 0.8. The expression of GST/*bglA* gene fusion was induced by adding IPTG to a final concentration of 1mM. The culture was grown for additional 3 hours and then harvested by centrifugation. Lysis of harvested bacteria was obtained by using a French® pressure cell press and purification of the induced fusion protein and removal of GST was done as specified by the vendor (Pharmacia; GST Gene Fusion System Manual, Third Edition, Revision 1). The concentration of the purified BglA protein was determined according to the BCA Protein Assay from PIERCE (Pockford, USA).

2.8 β-glucosidase activity on ONPG and esculin

Purified BglA polypeptide was measured for β-glucosidase activity and for determination of Michaelis constant K_M using o-nitrophenyl β-D-galactopyranoside (ONPG) as substrate as previously described for β-galactosidase activity (Phillips 1994). Briefly, the purified BglA protein was added in a concentration of 40ng/µl to a final volume of 1 ml Z-buffer (0.1 M sodium phosphate pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, 50 mM mercaptoethanol) containing ascendant (by steps of 0.2 mg/ml until 4 mg/ml) concentrations of ONPG. The solution was incubated at 28°C for 30 minutes and the reaction was terminated by addition of Na_2CO_3. The optical density of the solution was determined in a spectrophotometre (Pharmacia) at 420 nm. Determination of the maximal velocity V_{max} and the Michaelis constant K_M were done in a plot of the reaction velocity as a function of the substrate concentration and a double-reciprocal plot of enzyme kinetics (Lineweaver-Burk plot), respectively.

Esculinase- (β-glucosidase-) activity from *Listeria* strains was carried out with modified Oxford agar plates. Oxford agar base was supplemented only with esculin (Sigma) and ferric ammonium citrta (Merck), lacking all of the other recommended chemicals as described recently for the specific isolation of *L. monocytogenes* (Curtis et al. 1989). The agr plates were incubated at 37°C for variable time periods. Esculinase- (β-

glucosidase-) positive strains showed a black halo around bacterial colonies.

2.9 In vitro invasion assay

PtK_2 cells (ATCC CCL 56) were cultured in minimum essential medium 5MEM; Gibco) supplemented with 8% fetal calf serum, glutamine, and nonessential amino acids in the absence of antibiotics and were infected with both wild-type EGD and Δ*bglA*2 strains as described (Domann et al. 1997).

2.10 Mouse virulence assay

The mouse bioassay was performed as described by Nichterlein et al. (Nichterlein 1994).

2.11 Nucleotide sequence accession number

The nucleotide sequence data reported in this publication have been submitted to EMBL 5Cambridge, United Kingdom) and assigned the accession number Y11532 (*Listeria monocytogenes bglA* gene).

3. RESULTS

3.1 Cloning and sequencing of the *Listeria monocytogenes bglA* gene

A gene library comprising listerial DNA inserts cloned into the pAULA vector (Schäferkordt et al. 1998) was transformed into the *bglB* mutant strain MA 223 (Mahadevan et al. 1987a) and colonies selected for fermentation properties on arbutin-containing MacConkey agar plates. Several colonies showing weak activities were obtained, all of which were found to harbor a 7.5 kb *Hin*dIII insert. Nucleic acid sequencing of subclones obtained from this insert indicated the presence of regions with strong sequence homologies to bacterial β-glucosidases, enzymes that are known to hydrolyze the glucosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. We localized the corresponding *bgl* gene from *L. monocytogenes* to a 2423 bp long *Mun*I restriction endonuclease DNA fragment from plasmid pAUL-44 and determined its entire nucleic acid sequence (Fig. 1). An open reading frame, designated *bglA* was detected between positions 832 and 2247 of the sequence. It was preceded by a putative ribosome binding site (RBS) located 8 bp upstream of its initiation codon ATG. Additionally, a sequence of dyad symmetry resembling a rho-independent terminator (Platt 1986) was located immediately downstream of the termination codon suggesting that this gene was transcribed monocistronically. The stability of the stem-loop structure was predicted with a free energy of ΔG = -23.7 kcal/mol (Zuker 1989). The 5' upstream region of the *bglA* gene exhibited a sequence motif, termed ribonucleic antiterminator (RAT), which is strongly conserved in sequence and in position in the leader regions of the levansucrase gene *sacB* and the sucrase operon *sacPA* from *Bacillus subtilis* and the β-glucoside operon *bgl* from *Escherichia coli* (Aymerich and Steinmetz 1992). The strongly conserved RAT sequence in the upstream region of *bglA* was 31 bp long extending from positions 704 to 734 and overlapped with an extended stem-loop structure which spanned nucleotides 731 to 789 (Fig. 1). The predicted stability (Zuker 1989) of the proposed RAT structure (ΔG = -12.5 kcal/mol) was much lower than that of the associated terminator structure (ΔG= -30.7 kcal/rnol).

The reading frame encoding the putative β-glucosidase *BglA* comprised 471 amino acid residues with a calculated molecular mass of 53,646. Two potential signal peptidase II cleavage sites at the N-terminal end which were in close

agreement with the (-3-1) rule (Von Heijne 1984) were located between amino acid residues 18/19 and 19/20 (Fig. 1). The isoelectric point (pI) of the *bglA* gene product was calculated to be 4.73, indicating that BglA was an acidic protein. The upstream region of the *bglA* gene encodes a proline-rich protein with 399 amino acid residues and a calculated molecular weight of 43 kDa, which harbors a signal peptide and a putative membrane anchor. It exhibited weak sequence homologies to the fibronectin binding protein of *Staphylococcus aureus*. The downstream region encodes a small protein with 16 kDa which showed strong sequence homologies to the probable thiamin-phosphate pyrophosphorylases (EC 2.5.1.3) from *Helicobacter pylori and Haemophilus influenzae* (data not shown).

3.2 Homologies and phylogenetic tree of β-glucosidases from several microorganisms

The primary amino acid sequence of the *bglA* gene product was used to search protein data bases for homologous polypeptides employing BLASTP algorithm (Altschul et al. 1990) within the HUSAR environment. Homologies to BglA from *L. monocytogenes* were found among members of the tribes of eucaria (plants and humans) and bacteria with the strongest homologies to bacterial β-glucosidases (fig. 2). A phylogenetic tree for these β-glucosidases was generated and is depicted (data not shown). An unexpected result was that BglA from *L. monocytogenes* built up its own branch, along with AbgA from *Clostridium longisporum* (Brown and Thomson 1998). Of the four main branches of this phylogentic tree derived, this branch seems to be the most ancient. Based on the algorithm of the software of MEGALIGN from DNASTAR the age was calculated to be more than 500 million years. Despite the distances among these β-glucosidases the two conserved catalytic residues, a nucleophile and an acid catalyst (proton donor), are highly conserved (data not shown).

3.3 Detection of the *bglA* gene product

To identify the gene product encoded by the cloned *bglA* gene in *E. coli*, a PCR product with the oligonucleotides [E] 5'-TCAACGGTAGTAGAA GCTGATTC-3' and [F] 5'-TCAAAAGCGGATCCAGAGCCAGTG AG-3' was generated from plasmid pAUL-44. This amplicon contained the reading frame corresponding to the *bglA* gene and flanking sequences and was cloned into pCRII vector plasmid (pCRII-*bglA*) and was transformed into the *E. coli* CSH26ΔF6 maxicell strain. Polypeptides encoded by pCRII-*bglA* and the vector plasmid pCRII were detected by radioactive labeling with [^{35}S]methionine. A unique 54-kDa polypeptide was expressed in the strain carrying pCRII-*bglA*. An additional faint band with a calculated molecular weight of 38 kDa, most likely a degradation product of the BglA protein, was also detected (Fig. 3, lane 2). Syntheses of all of the other identifiable proteins detected are directed by the vector plasmid sequences (Fig. 3, lanes 1 and 2). As determined theoretically and experimentally the molecular weight of the *bglA* gene product was 54-kDa.

3.4 Purification of the BglA polypeptide as a recombinant GST fusion protein in *E. coli*

To study the biochemical characteristics of the BglA polypeptide from *L. monocytogenes* we expressed it in *E. coli* and purified it employing the recombinant GST gene fusion system from Pharmacia. The expression plasmid

pGEX-6P-1 allows the C-terminal fusion of a protein of interest to gluthatione S-transferase (GST) and a PreScission™ protease cleavage site. A specific 1.6-kb DNA fragment encoding the mature BglA polypeptide without ist 19 amino acid residues long signal peptide was amplified from template plasmid pAUL-44 with the oligonucleotides GST/*bglA*-*Bam*HI and GST/*bglA*-*Mun*I and was cloned into the *Bam*HI and *Eco*RI restriction sites of the multiple cloning site of plasmid pGEX-6P-1 generating plasmid pGST-*bglA*. The fusion of the GST-gene and the *bglA*-gene and the correct nucleotide sequence of plasmid pGST-*bglA* was confirmed by nucleic acid sequencing (data not shown). This cloning strategy enabled us to fuse the mature BglA polypeptide to GST and to purify it as outlined by the vendor (Pharmacia). The fusion protein was composed of GST (29 kDa), a PreScission™ protease cleavage site and the mature BglA polypeptide (51.6 kDa) with a calculated entire molecular weight of 80.6 kDa (Fig. 4A, lane 2). After cleavage of the GST- BglA fusion protein with the GST-fused PreScission™ protease both GST and the protease were sedimented with glutathione sepharose 4B and pure BglA was recovered from the supernatant (Fig. 4B, lane 1). A yield of 1.6 mg of purified BglA per liter bacterial culture volume was obtained using this method.

3.5 Enzymatic activity on o-nitrophenyl-β-D-galactopyranoside (ONPG)

To determine the Michaelis constant K_M of the purified β-glucosidase on ONPG, varying concentrations of the chromogenic substrate ranging from zero to 4 mg/ml in ascending steps of 0.2 mg/ml were used (Fig. 5)(Phillips 1994). A saturation and the maximal velocity of the reaction was obtained at a concentration of 2.8 mg/ml of ONPG and at an optical density at A_{420} of 0.3. The Michaelis constant of BglA from *L. monocytogenes* under the conditions described in materials and methods was determined by a Lineweaver-Burk plot (56) to be 9.71×10^{-4} M (~ 1×10^{-3} M).

3.6 Presence of the *bglA* gene in *Listeria* species

An isogenic *bglA* deletion mutant was constructed to examine the role of the *bglA* gene in the physiology and virulence of *L. monocytogenes.* In order to identify flanking restriction endonuclease sites that could be used to generate the mutant, and to verify the colinearity of the DNA insert in pAUL-44, chromosomal DNA was digested with restriction endonucleases *Hind*III, *Eco*RV, *Mun*I, and *Xba*I, respectively, electrophoresed, and immediately transferred to a nylon membrane. A *bglA*-specific DNA fragment of 0.98 kb was amplified from plasmid pAUL-44 as the template with the oligonucleotide pair [A] and [B] and served as the specific [α-^{32}P]dATP labeled probe in hybridization experiments. Restriction endonuclease mapping of the pAUL-44 recombinant had previously indicated that the *bglA* gene was located on a 7.5-kb *Hind*III and a 2.4-kb *Mun*I restriction endonuclease fragment respectively; exactly these results were obtained using the mentioned enzymes. Additional restriction endonuclease analysis with restriction enzymes *Eco*RV and *Xba*I enabled us to locate *bglA* on a 9.0-kb *Eco*RV and a 13.8-kb *Xba*I chromosomal DNA fragment of *L. monocytogenes*. Overexposure of the autoradiogram revealed additional visible bands which were either of a higher or a lower molecular size than those described above. The digestion of the chromosomal DNA with *Eco*RV restriction enzyme revealed one faint additional band with a

calculated molecular size of approximately 10.1 kb. The digestion with restriction enzyme *Mun*I revealed additional restriction fragments with calculated molecular sizes of approximately 0.75, 4.3, 12.0, and 14.5 kb. The hybridization experiments performed indicate that the genome of *L. monocytogenes* may harbor further nucleic acid sequences with sequence homologies to the *bglA* gene (data not shown).

The *bglA* deletion mutant was constructed as described in the Materials and Methods section. Since esculin fermentation is a diagnostic marker for *Listeria* spp. we compared the ability of the Δ*bgl2* mutant to ferment this sugar compared to the wild-type EGD on modified Oxford agar plates. No difference was observed indicating that BglA is dispensable for the hydrolysis of esculin and that *L. monocytogenes* must harbor at least one other β-glucosidase. No differences were also visible between the mutant and the wild type strain in tissue culture assays assessing adhesion, invasion, intracellular motility or cell-to-cell spread or in the mouse model of infection (data not shown).

In order to determine the presence of the *bglA* gene in pathogenic and nonpathogenic *Listeria* species, chromosomal DNA from *L. monocytogen*es, *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welshimeri*, and *L. grayi* were used as a template to amplify *bglA*-specific DNA fragments. The oligonucleotides [A] and [B] were used to amplify an internal 980-bp DNA fragment from the coding sequence of the *bglA* gene. In addition, the oligonucleotides [E] and [F] flanking the *bglA* gene were used to amplify a 2.12-kb specific DNA fragment. The 980-bp specific nucleic acid sequence representing the coding sequence of *bglA* was also present in the nonpathogenic strains *L. seeligeri*, *L. innocua*, and *L. welshimeri* (data no shown). However, for *L. grayi*, a 3.0 kb amplicon was detected instead of the expected 980 bp fragment. No *bglA* specific PCR product was detectable in the pathogenic strain *L. ivanovii* and the no pathogenic strain *L. grayi*, indicating that those strains lack the *bglA* gene (data no shown). The 2.12-kb PCR product, specific for the *bglA* gene and its flanking regions from *L. monocytogenes*, was not detectable in all of the other *Listeria* strains, indicating that the flanking regions were not identical (data no shown).

3.7 Detection of *bglA*-specific transcripts in *L. monocytogenes*

We isolated the total RNA of PtK_2 host cells infected with the wild-type EGD and examined for *bglA*- and *hly*-specific transcripts employing RT-PCR. No *bglA*-specific transcript was detectable, whereas a listeriolysin-specific transcript, which is intracellular expressed and served as the positive control, was clearly detectable (data not shown). Hence, the *bglA* gene was not expressed intracellular in infected PtK_2 host cells.

RAT sequences present in the upstream region of the *bglA* gene in many species have been demonstrated to prevent transcription in the absence of an inducer. To identify putative inducers of the *bglA* gene we isolated the mRNA from *L. monocytogenes* grown in LB broth in the presence of the sugars xylose, sucrose, salicine, rhamnose, mannoside, glucose, galactose, cellobiose, or arbutin and looked for *bglA*- and *hly*-specific transcripts by RT-PCR (data not shown). This essay shows faint *bglA*-specific transcripts in the presence of xylose, mannoside, glucose and arbutin, indicating that under these conditions these sugars served, however, as weak inducers. No transcript was detected in the presence of sucrose, salicine,

rhamnose, galactose and cellobiose and in the LB broth without sugars (data not shown).

4. DISCUSSION

A gene encoding a β-glucosidase has been isolated from the bacterial pathogen *Listeria monocytogenes*. The *bglA*-gene encodes a protein of 54 kDa with enzymatic activity when assessed with ONPG as substrate. The *bglA*-gene was highly conserved in all *Listeria* species excepting the animal pathogenic strain *L. ivanovii* and *L. grayi*. In contrast no PCR amplicons were detected with primers derived from the flanking regions of the *bglA* gene in other *Listeria* species. The finding that oligonucleotides internal to the *bglA* gene gave amplicons for several species but these strains were negative when probed with *bglA* flanking oligonucleotides suggest that *bglA* has a different chromosomal location in *Listeria* strains, which are positive for the *bglA* gene. In *L. monocytogenes* the *bglA* gene is physically linked between a proline-rich protein with weak sequence homologies to the fibronectin-binding protein from *Staphylococcus aureus* and a probable thiamin-phosphate pyrophosphorylase from *Helicobacter pylori* and *Haemophilus influenzae* (data not shown).

The 5'-region of the *bglA* gene harbors a sequence motif which is strongly conserved in sequence and in position in the leader regions of the β-glucosidase operon *bgl* from *E. coli*, the levansucrase gene *sacB*, and the sucrase operon *sacPA* from *Bacillus subtilis*, termed ribonucleic antiterminator (RAT) (Crutz et al. 1990; Mahadevan and Wright 1987b; Schnetz and Rak 1988; Shimotsu and Henner 1986; Steinmetz et al. 1989). In the *E. coli bgl*-system the antiterrninator BglG is an RNA binding protein which enables, in its active dephosphorylated form, transcription of genes with RAT-like sequences in their promoter regions. In the *bgl*-operon from *E. coli* and in a newly discovered β-glucoside utilization system (*licH*, *licR*) from *B. subtilis* the genes encoding an antiterminator are physically linked to the β-glucosidase genes (Houman et al. 1990; Tobisch et al. 1997). However, induction of transcription also occurs in Trans when antiterminator and target sequences are physically separated as for example with the *bglPH* operon of *B. subtilis* (Le-Coq et al. 1995). Such RNA binding proteins are members of a growing BglG family of transcriptional antiterminators which have been described for *B. subtilis licR*, *licT*, *sacT*, and *sacY*, for *Erwinia chrysanthemi arbG*, and for *Lactococcus lactis bglR* (Bardowski et al. 1994; Debarbouille et al. 1990; El-Hassouni et al. 1992). Control of expression of the *L. monocytogenes bglA* gene is likely to resemble those already known for the bgl genes in *E. coli* and *B. subtilis*.

Based on amino acid sequence comparisons BglA from *L. monocytogenes* is a member of the family one glycosyl hydrolases which occur in different living organisms. The strongest homologies were found to bacterial β-glucosidases from gram-positive and gram-negative bacteria. A phylogenetic tree revealed that these β-glucosidases share a common ancestor which ramifies into four major branches approximately 800 million years ago. An intriguing result is that most of the enzymes are comparatively young β-glucosidases and that BglA from *L. monocytogenes* belongs, along with the recently discovered AbgA from *Clostridium longisporum*, to the oldest branch in this tree. However, despite the distances among these β-glucosidases the two conserved catalytic residues, a nucleophile and an acid catalyst (proton donor), are highly conserved (Fig. 2B)(Henrissat et al. 1995). Recently,

Sanz-Aparicio and colleagues determined the crystal structure of BglA from *B. polymyxa* providing detailed insights into the catalytic activity in the family one type of glycosyl hydrolases (Sanz-Aparicio 1998). The data presented here indicates that BglA from *L. monocytogenes* is likely to exhibit the same catalytic mechanism which enables it to hydrolyze glycosidic bonds from several substrates.

To examine if BglA is responsible for the diagnostic esculinase activity exhibited by *L. monocytogenes*, we generated a Δ*bglA* mutant strain and inoculated Oxford agar plates employing the esculin-ferric ammonium citrate indicator system (Curtis et al. 1989; Edberg et al. 1977). Like the wild-type EGD, Δ*bglA* mutant strain showed black halos around bacterial colonies indicating β-glucosidase- (esculinase-) activity (data not shown). In order to investigate potential inducers of the *bglA* gene, we cultivated wild-type EGD in LB culture broth where no *bglA*-specific transcript was detectable. Only in the presence of xylose, mannoside, glucose and arbutin in the growth medium we were able to detect expression of the *bglA*-gene. For routine biochemical differentiation of species within the genus *Listeria*, acid production from mannoside and from rhamnose is used to distinguish *L. monocytogenes* from other species (Swaminathan et al. 1995). The presence of mannoside revealed weak and that of rhamnose no transcription of the *bglA* gene, suggesting that more than one β-glucosidase gene is required to exhibit these differentiating properties.

Altogether, these results indicate the presence of more β-glucosidases within the genome of *L. monocytogenes*. In another gram-positive bacterium, *B. subtilis*, several genes and operons involved in β-glycoside utilization are known (Glaser et al. 1993; Leimeister-Wächter and Chakraborty 1989; Murphy et al. 1984; Tobisch et al. 1997; Zhang and Aronson 1994), and the genome project of *E. coli* revealed also several genes which are possibly members of the family one glycosyl hydrolases (Blattner et al. 1997). Employing a *bglA*-specific DNA probe and hybridization under low stringency conditions we detected DNA fragments on the genome of *L. monocytogenes* which may encode additional β-glucosidases. Indeed, another β-glucosidase gene has been detected recently on the chromosome of *L. monocytogenes*: a 6-phospho-β-D-glucosidase gene is located upstream of a new internalin operon (Dramsi et al. 1997).

Since the Δ*bglA* mutation was phenotypically not detectable we wished to know if the *bglA* gene is functional and indeed encodes for an enzymatically active β-glucosidase. Maxicell analysis confirmed that the cloned *bglA* gene encodes a polypeptide with a molecular mass of 54 kDa. For detection of biological activity we purified the mature protein employing the GST gene fusion system (Pharmacia) and used the established β-galactosidase assay for hydrolysis of ONPG to o-nitrophenol and galactose (Phillips 1994). The recombinant BglA polypeptide exhibited enzymatic activity towards this substrate indicating that it is able to hydrolyze the non-phosphorylated form of the glycoside. Other glycosidases are phosphoglycosidases which act only on the phosphorylated form of the glycoside (Smibert and Krieg 1994). We determined the K_M value of 9.71×10^{-4} M ($\sim 1 \times 10^{-3}$ M) for the *bglA* gene product on the substrate ONPG. Albeit the K_M values of enzymes range widely, for most enzymes K_M lies between 10^{-1} and 10^{-7} M, which corresponds very well to the K_M of the BglA polypeptide (Stryer, L. 1988).

Recently, it has been shown that the β-glucosidase operon of a pathogenic strain of *Escherichia coli* is induced during bacterial infection of the mouse liver (Khan and Isaacson 1998). To search for a possible effect of the *bglA* expression during intracellular growth we examined *bglA* transcription following infection of tissue culture cells and mice. In PtK_2 host cells the mutation did not influence entry, escape from the phagosome, intracellular motility and cell-to-cell spread and the generation time for intracellular replication. No *bglA*-specific transcripts were detected during intracellular growth indicating that no induction had occurred. Also, no significant differences were detected in the mouse model of infection compared to the wild-type. From these studies we conclude that the *bglA* gene is dispensable for intracellular growth and survival.

The physical identification of a β-glucosidase and information regarding its inducibility will contribute to the overall understanding of the physiology and the ecological niches used by this bacterium. Our current studies are aimed at identifying the antitermination protein required for regulation of *bglA* transcription. The identification and isolation of additional β-glucosidases in *L. monocytogenes* should provide us with information as to how the expression of virulence factors in this species is regulated by the availability of these sugars.

5. ACKNOWLEDGMENT

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7. FIGURES AND LEGENDS

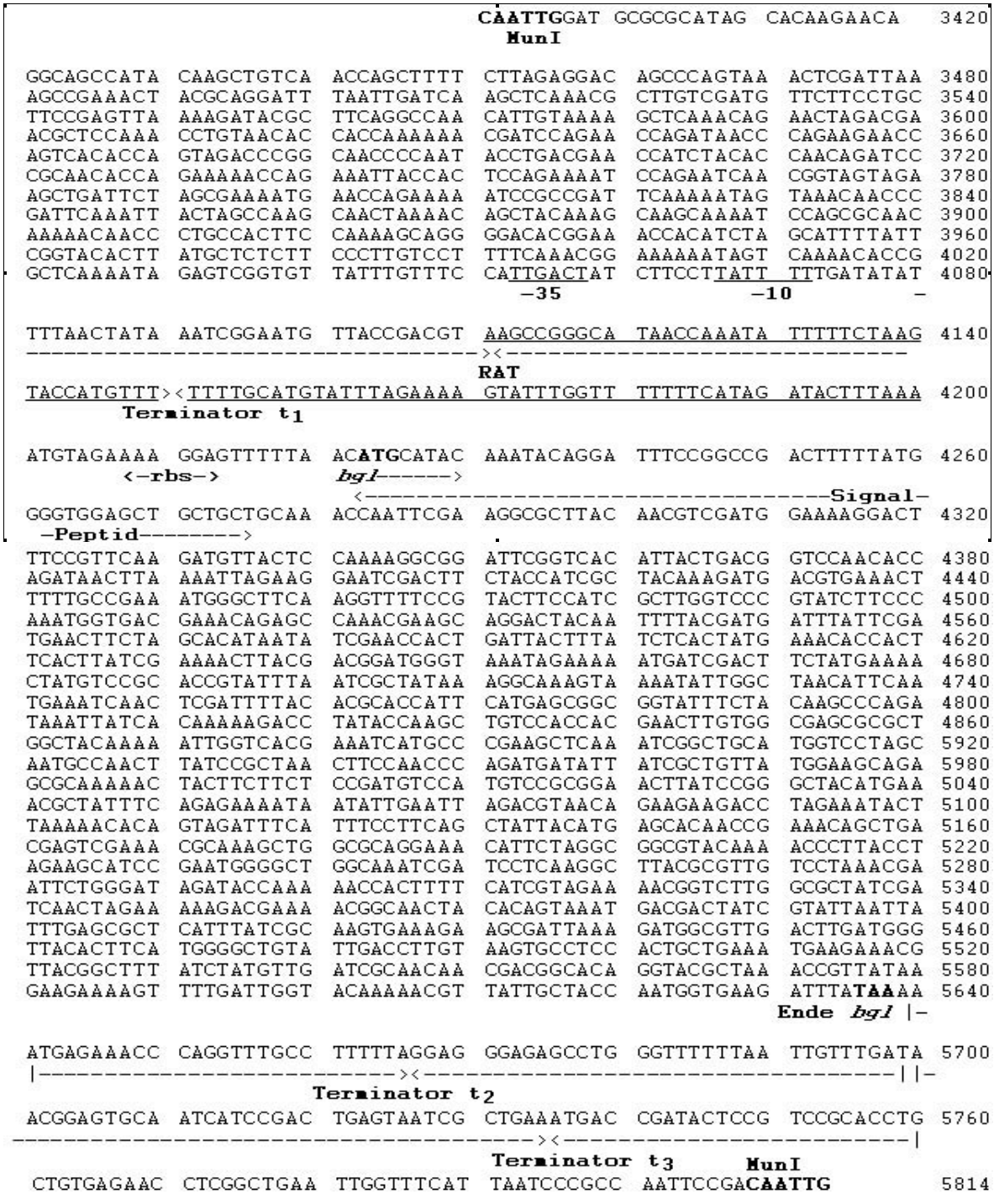

Figure 1: Nucleotide sequence of the 2423-bp long *MunI* endonuclease restriction fragment of *L. monocytogenes* encoding the gene *bglA* and the deduced amino acid sequence of the corresponding gene product, BglA. The putative promoter region is indicated with -35 and -10 and the ribosome binding site (RBS) is in boldface type and underlined. The conserved regulatory motif for putative binding of a ribonucleic antiterminator is indicated with + and RAT sequence. The RAT sequence overlaps with an inverted repeat sequence which is indicated by convergent arrows above the sequence and by terminator one. The 18/19-arnino-acid-long signal peptide is in italics and the two arrows indicate the putative signal peptidase cleavage sites. The putative rho-independent terminator is indicated by convergent arrows and named terminator two.

```
bgllmo.pep   : Listeria monocytogenes
bglbsu.pep   : Bacillus subtilis
bglbpo.pep   : Bacillus polymyxa
bglbci.pep   : Bacillus circulans
bgllla.pep   : Lactobacillus lactis
bgllca.pep   : Lactobacillus casei
bglsau.pep   : Staphylococcus aureus
bglcth.pep   : Clostridium thermocellum
bgleco.pep   : Escherichia coli
bglech.pep   : Erwinia chrysanthemi
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bgllmo.pep       MHTNTG-FPADFLWGGAAAANQFEGAYNVDGKGLSVQDVTPKGG--------FGHITDGP
bglbsu.pep       MSSNEKRFPEGFLWGGAVAANQVEGAYNEGGKGLSTADVSPNGI--------MSPFDESM
bglbpo.pep       --MTIFQFPQDFMWGTATAAYQIEGAYQEDGRGLSIWDTFAHTP--------------GK
bglbci.pep       --MSIHMFPSDFKWGVATAAYQIEGAYNEDGRGMSIWDTFAHTP--------------GK
bgllla.pep       ---MTKTLPKDFIFGGATAAYQAEGATHTDGKGPVAWDKYLEDN--------------YW
bgllca.pep       ---MSKQLPQDFVMGGATAAYQVEGATKEDGKGRVLWDDFLDKQ--------------GR
bglsau.pep       ---MTKTLPEDFIFGGATAAYQAEGATNTDGKGRVAWDTYLEEN--------------YW
bglcth.pep       --MSKITFPKDFIWGSATAAYQIEGAYNEDGKGESIWDRFSHTP--------------GN
bgleco.pep       ----MSVFPESFLWGGALAANQSEGAFREGDKGLTTVDMIPHGEHRMAVKLGLEKRFQLR
bglech.pep       ---MSNPFPAHFLWGGAIAANQVEGAYLTDGKGLSTSDLQPQGI--------FGEIVTRQ
                         *  *  * * ** * ***     .*    *

bgllmo.pep       TPDN-LKLEGIDFYHRYKDDVKLFAEMGFKVFRTSIAWSRIFPNGDETEPNEAGLQFYDD
bglbsu.pep       TSLN-LYHNGIDFYHRYKEDIALFAEMGFKAFRTSIAWTRIFPNGDEEEPNEEGLRFYDD
bglbpo.pep       VFNGDNGNVACDSYHRYEEDIRLMKELGIRTYRFSVSWPRIFPNGDG-EVNQEGLDYYHR
bglbci.pep       VKNGDNGNVACDSYHRVEEDVQLLKDLGVKVYRFSISWPRVLPQGTG-EVNRAGLDYYHR
bgllla.pep       YT----AEPASDFYHKYPVDLELAEEYGVNGIRISIAWSRIFPTGYG-EVNEKGVEFYHK
bgllca.pep       FK----PDPAADFYHRYDEDLALAEKYGHQVIRVSIAWSRIFPDGAG-EVEPRGVAFYHK
bglsau.pep       YT----AEPASDFYNRYPVDLELSEKFGVNGIRISIAWSRIFPNGYG-EVNPKGVEYYHK
bglcth.pep       IADGHTGDVACDHYHRYEEDIKIMKEIGIKSYRFSISWPRIFPEGTG-KLNQKGLDFYKR
bgleco.pep       DDEFYPSHEATDFYHRYKEDIALMAEMGFKVFRTSIAWSRLFPQGDEITPNQQGIAFYRS
bglech.pep       PGDSGIKDVAIDFYHRYPQDIALFAEMGFTCLRISIAWTRIFPQGDEAEPNEAGLAFYDR
                            * *..   *. .    *    * *..* *. * *        *. .*

bgllmo.pep       LFDELLAHNIEPLITLSHYETPLHLSKTYDGWVNRKMIDFYENYVRTVFNRYKGKVKYWL
bglbsu.pep       LFDELLKHHIEPVVTISHYEMPLGLVKNYGGWKNRKVIEFYERYAKTVFKRYQHKVKYWM
bglbpo.pep       VVDLLNDNGIEPFCTLYHWDLPQALQDA-GGWGNRRTIQAFVQFAETMFREFHGKIQHWL
bglbci.pep       LVDELLANGIEPFCTLYHWDLPQALQDQ-GGWGSRITIDAFAEYAELMFKELGGKIKQWI
bgllla.pep       LFAECHKRHVEPFVTLHHFDTPEALHSN-GDFLNRENIEHFIDYAAFCFEEFP-EVNYWT
bgllca.pep       LFADCAAHHIEPFVTLHHFDTPERLHEA-GDWLSQEMLDDFVAYAKFCFEEFS-EVKYWI
bglsau.pep       LFAECHKRHVEPFVTLHHFDTPEVLHKD-GDFLNRKTIDYFVDYAEYCFKEFP-EVKYWT
bglcth.pep       LTNLLLENGIMPAITLYHWDLPQKLQDK-GGWKNRDTTDYFTEYSEVIFKNLGDIVPIWF
bgleco.pep       VFEECKKYGIEPLVTLCHFDVPMHLVTEYGSWRNRKLVEFFSRYARTCFEAFDGLVKYWL
bglech.pep       LFDELAKYGIQPLVTLSHYEMPYGLVEKHGGWGNRLTIDCFERYARTVFARYRHKVKRWL
                 .        . *  *. * . *  *         .     .  .    *      .  *
```


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bgllmo.pep       MHTNTG-FPADFLWGGAAAANQFEGAYNVDGKGLSVQDVTPKGG--------FGHITDGP
bglbsu.pep       MSSNEKRFPEGFLWGGAVAANQVEGAYNEGGKGLSTADVSPNGI--------MSPFDESM
bglbpo.pep       --MTIFQFPQDFMWGTATAAYQIEGAYQEDGRGLSIWDTFAHTP--------------GK
bglbci.pep       --MSIHMFPSDFKWGVATAAYQIEGAYNEDGRGMSIWDTFAHTP--------------GK
bgllla.pep       ---MTKTLPKDFIFGGATAAYQAEGATHTDGKGPVAWDKYLEDN--------------YW
bgllca.pep       ---MSKQLPQDFVMGGATAAYQVEGATKEDGKGRVLWDDFLDKQ--------------GR
bglsau.pep       ---MTKTLPEDFIFGGATAAYQAEGATNTDGKGRVAWDTYLEEN--------------YW
bglcth.pep       --MSKITFPKDFIWGSATAAYQIEGAYNEDGKGESIWDRFSHTP--------------GN
bgleco.pep       ----MSVFPESFLWGGALAANQSEGAFREGDKGLTTVDMIPHGEHRMAVKLGLEKRFQLR
bglech.pep       ---MSNPFPAHFLWGGAIAANQVEGAYLTDGKGLSTSDLQPQGI--------FGEIVTRQ
                         *  *  * * ** * ***     .*    *
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bgllmo.pep          TPDN-LKLEGIDFYHRYKDDVKLFAEMGFKVFRTSIAWSRIFPNGDETEPNEAGLQFYDD
bglbsu.pep          TSLN-LYHNGIDFYHRYKEDIALFAEMGFKAFRTSIAWTRIFPNGDEEEPNEEGLRFYDD
bglbpo.pep          VFNGDNGNVACDSYHRYEEDIRLMKELGIRTYRFSVSWPRIFPNGDG-EVNQEGLDYYHR
bglbci.pep          VKNGDNGNVACDSYHRVEEDVQLLKDLGVKVYRFSISWPRVLPQGTG-EVNRAGLDYYHR
bgllla.pep          YT----AEPASDFYHKYPVDLELAEEYGVNGIRISIAWSRIFPTGYG-EVNEKGVEFYHK
bgllca.pep          FK----PDPAADFYHRYDEDLALAEKYGHQVIRVSIAWSRIFPDGAG-EVEPRGVAFYHK
bglsau.pep          YT----AEPASDFYNRYPVDLELSEKFGVNGIRISIAWSRIFPNGYG-EVNPKGVEYYHK
bglcth.pep          IADGHTGDVACDHYHRYEEDIKIMKEIGIKSYRFSISWPRIFPEGTG-KLNQKGLDFYKR
bgleco.pep          DDEFYPSHEATDFYHRYKEDIALMAEMGFKVFRTSIAWSRLFPQGDEITPNQQGIAFYRS
bglech.pep          PGDSGIKDVAIDFYHRYPQDIALFAEMGFTCLRISIAWTRIFPQGDEAEPNEAGLAFYDR
                               * *..   *. .    *    * *..* *. * *        *. .*

bgllmo.pep          LFDELLAHNIEPLITLSHYETPLHLSKTYDGWVNRKMIDFYENYVRTVFNRYKGKVKYWL
bglbsu.pep          LFDELLKHHIEPVVTISHYEMPLGLVKNYGGWKNRKVIEFYERYAKTVFKRYQHKVKYWM
bglbpo.pep          VVDLLNDNGIEPFCTLYHWDLPQALQDA-GGWGNRRTIQAFVQFAETMFREFHGKIQHWL
bglbci.pep          LVDELLANGIEPFCTLYHWDLPQALQDQ-GGWGSRITIDAFAEYAELMFKELGGKIKQWI
bgllla.pep          LFAECHKRHVEPFVTLHHFDTPEALHSN-GDFLNRENIEHFIDYAAFCFEEFP-EVNYWT
bgllca.pep          LFADCAAHHIEPFVTLHHFDTPERLHEA-GDWLSQEMLDDFVAYAKFCFEEFS-EVKYWI
bglsau.pep          LFAECHKRHVEPFVTLHHFDTPEVLHKD-GDFLNRKTIDYFVDYAEYCFKEFP-EVKYWT
bglcth.pep          LTNLLLENGIMPAITLYHWDLPQKLQDK-GGWKNRDTTDYFTEYSEVIFKNLGDIVPIWF
bgleco.pep          VFEECKKYGIEPLVTLCHFDVPMHLVTEYGSWRNRKLVEFFSRYARTCFEAFDGLVKYWL
bglech.pep          LFDELAKYGIQPLVTLSHYEMPYGLVEKHGGWGNRLTIDCFERYARTVFARYRHKVKRWL
                    .        . *  *. * . *  *         .     .  .    *      .  *

bgllmo.pep          TFNEINSILHAPFMSGGISTSPDKLSQKDLYQAVHHELVASALATKIGHEIMPEAQIGCM
bglbsu.pep          TFNEINVVLHAPFTGGGLVFEEGENKLNAMYQAAHHQFVASALAVKAGHDIIPDSKIGCM
bglbpo.pep          TFNEPWCIAFLSNMLGVHAPGLT--NLQTAIDVGHHLLVAHGLSVRRFRELGTSGQIGIA
bglbci.pep          TFNEPWCMAFLSNYLGVHAPGNK--DLQLAIDVSHHLLVAHGRAVTLFRELGISGEIGIA
bgllla.pep          TFNEIGPIGDGQYLVGKFPPGIKY-DLAKVFQSHHNMMVSHARAVKLYKDKGYKGEIGVV
bgllca.pep          TINEPTSMAVQQYTTGTFPPAESG-RFDKTFQAEHNQMVAHARIVNLYKSMQLGGQIGIV
bglsau.pep          TFNEIGPIGDGQYLVGKFPPGIKY-DFEKVFQSHHNMMVAHARAVKLFKDGGYKGEIGVV
bglcth.pep          THNEPGVVSLLGHFLGIHAPGIK--DLRTSLEVSHNLLLSHGKAVKLFREMNIDAQIGIA
bgleco.pep          TFNEINIMLHSPFSGAGLVFEEGENQDQVKYQAAHHQLVASALATKIAHEVNPQNQVGCM
bglech.pep          TFNEINMSLHAPFTGVGLPPDSDK---AAIYQAIHHQLVASARAVKACHDMIPDAQIGNM
                    * **                              *.  ..        .       .*

bgllmo.pep          VLAMPTYPLT-SNPDDIIAVMEAE-RKNYFFSDVHVRGTYPGYMKRYFR-----ENNIEL
bglbsu.pep          IAATTTYPMT-SKPEDVFAAMENE-RKTLFFSDVQARGAYPGYMKRYLA-----ENNIEI
bglbpo.pep          PNVSWAVPYS-TSEEDKAACARTISLHSDWFLQPIYQGSYPQFLVDWFA-----EQGATV
bglbci.pep          PNTSWAVPYR-RTKEDMEACLRVNGWSGDWYLDPIYFGEYPKFMLDWYE-----NLGYKP
bgllla.pep          HALPTKYPYDPENPADVRAAELEDIIHNKFILDATYLGHYSDKTMEGVNHILAENGG-EL
bgllca.pep          HALQTVYPYS-DSAVDHHAAELQDALENRLYLDGTLAGEYHQETLALVKEILDANHQPMF
bglsau.pep          HALPTKYPFDPSNPEDVRAAELEDIIHNKFILDATYLGKYSRETMEGVQHILSVNGG-KL
bglcth.pep          LNLSYHYPAS-EKAEDIEAAELSFSLAGRWYLDPVLKGRYPENALKLYK-----KKGIEL
bgleco.pep          LAGGNFYPYS-CKPEDVWAALEKD-RENLFFIDVQARGTYPAYSARVFR-----EKGVTI
bglech.pep          LLGAMLYPLT-SKPEDVMESLHQN-REWLFFGDVQVRGAYPGYMHRYFR-----EQGITL
                           *       *                     * *

bgllmo.pep          DVTEEDLEILKN---TVDFISFSYYMSTTETADESKRK-------AGAG------NILGG
bglbsu.pep          EMAEGDEELLKEH--TVDYIGFSYYMSMAASTDPEELA-------KSGG------NLLGG
bglbpo.pep          PIQDGDMDIIGEP---IDMIGINYYSMSVNRFNP-----------EAG-------FLQSE
bglbci.pep          PIVDGDMELIHQP---IDFIGINYYTSSMNRYNPG----------EAGG------MLSSE
bgllla.pep          DLRDEDFQALDAAKDLNDFLGINYYMSDWMQAFDGETEIIHNGKGEKGSSKYQIKGVGRR
bgllca.pep          QSTPQEMKAIDEAAHQLDFVGVNNYFSKWLRAYHGKSETIHNGDGTKGSSVARLQGVGEE
bglsau.pep          NITDEDYAILDAAKDLNDFLGINYYMSDWMRGYDGESEITHNATGDKGGSKYQLKGVGQR
bglcth.pep          SFPEDDLKLISQP---IDFIAFNNYSSEFIKYDPS----------SESG------FSPAN
bgleco.pep          NKAPGDDEILKN---TVDFVSFSYYASRCASAEMNANN-------SSAA------NVVKS
bglech.pep          NITAQDKQDLKA---TVDFISFSYYMTGCVTTDEAQLE-------KTRG------NILNM
                         .   .       * .    *
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bgllmo.pep      VQNPYLEASEWGWQIDPQGLRVVLNEFWDRYQK--PLFIVENGLGAIDQLEKDENGN--Y
bglbsu.pep      VKNPYLKSSEWGWQIDPKGLRITLNTLYDRYQK--PLFIVENGLGAVDKVEED--G----
bglbpo.pep      EINMGLPVTDIGWPVESRGLYEVLHY-LQKYGN-IDIYITENGACINDEV-VN--G----
bglbci.pep      AISMGAPKTDIGWEIYAEGLYDLLRYTADKYGN-PTLYITENGACYNDGLSLD--G----
bgllla.pep      VAPDYVPRTDWDWIIYPEGLYDQIMRVKNDYPNYKKIYITENGLGYKDQFVD-------K
bgllca.pep      KLPDGIETTDWDWSIYPRGMYDILMRIHNDYPLVPVTYVTENGIGLKESLPEN--ATPDT
bglsau.pep      EFDVDVPRTDWDWMIYPQGLYDQIMRVVKDYPNYHKIYITENGLGYKDEFIES--E---K
bglcth.pep      SILEKFEKTDMGWIIYPEGLYDLLMLLDRDYGK-PNIVISENGAAFKDEIGSN--G----
bgleco.pep      LRNPYLQVSDWGWGIDPLGLRITMNMMYDRYQK--PLFLVENGLGAKDEFAAN--G----
bglech.pep      VPNPYLESSEWGWQIDPLGLRYLLNFLYDRYQK--PLFIVENGLGAKDKIEEN--G----
                          ..   *  .   *.    .       *       . ***    .

bgllmo.pep      TVNDDYRINYLSAHLSQVKEAIKDGVDLMGYTSWGCIDLVSASTAEMKKRYGFIYVDRNN
bglbsu.pep      TIQDDYRINYLRDHLIEARERIADGVELIGYTSWGPIDLVSASTAEMKKRYGFIYVDRDN
bglbpo.pep      KVQDDRRISYMQQHLVQVHRTIHDGLHVKGYMAWSLLDNFEWAEG-YNMRFGMIHVDFR-
bglbci.pep      RIHDQRRIDYLAMHLIQASRAIEDGINLKGYMEWSLMDNFEWAEG-YGMRFGLVHVDYD-
bgllla.pep      TVYDDGRIDYVKQHLEVLSDAIADGANVKGYFIWSLMDVFSWSNG-YEKRYGLFYVDFD-
bgllca.pep      VIEDPKRIDYVKKYLSAMADAIHDGANVKGYFIWSLQDQFSWTNG-YSKRYGLFFVDFP-
bglsau.pep      TVHDDARIDYVRQHLNVIADAIIDGANVKGYFIWSLMDVFSWSNG-YEKRYGLFYVDFE-
bglcth.pep      KIEDTKRIQYLKDYLTQAHRAIQDGVNLKAYYLWSLLDNFEWAYG-YNKRFGIVHVNFD-
bgleco.pep      EINDDYRISYLREHIRAMGGTIADGIPLMGYTTWGCIDLVSACTGEMSKRYGFVFVDRDD
bglech.pep      DIYDDYRIRYLNDHLVQVGEAIDDGVEVLGYTCWGPIDLVSASKAEMSKRYGFIYVDRDD
                  . *  ** *.      .       * **   .  *  *   *     .       *.*   *

bgllmo.pep      DGTGTLNRYKKKSFDWYKNVIATNGEDL--
bglbsu.pep      EGNGTFNRIKKKSFNWYQQVIATNGESL--
bglbpo.pep      ----TQVRTPKESYYWYRNVVSNNWLETRR
bglbci.pep      ----TLVRTPKDSFYWYKGVISRGWLDL--
bgllla.pep      ----TQERYPKKSAHWYKKLAETQVIE---
bgllca.pep      ----TQNRYIKQSAEWFKSVSETHIIPD--
bglsau.pep      ----TQERYPKKSAYWYKELAETKEIK---
bglcth.pep      ----TLERKIKDSGYWYKEVIKNNGF----
bgleco.pep      AGNGTLTRTHRKSFWWYKKVIASNGEDLE-
bglech.pep      AGHGSLERRRKKSFYWYQSVIASHGKTLTR
                     .  *   .  *  *.  .
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Figure 2: Comparison of BglA from *L. monocytogenes* with several β-glucosidases, amino acid sequence alignment of the conserved catalytic residues of the proton donor (acid catalyst) and nucleophile.

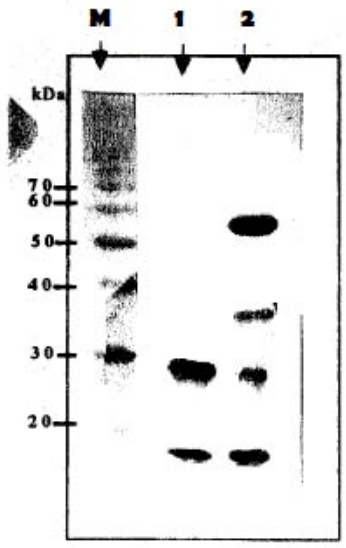

Figure 3: Autoradiograph of ^{35}S-labeled polypeptides encoded by pCRII (lane 1) and pCRII-*bglA* in maxicells. The 54-kDa polypeptide in lane 2 is the product of the *bglA* gene. An additional faint band with a calculated molecular weight of 38 kDa, most likely a degradation product of the BglA protein, was also detected (lane 2). Molecular mass standard is indicated kDa.

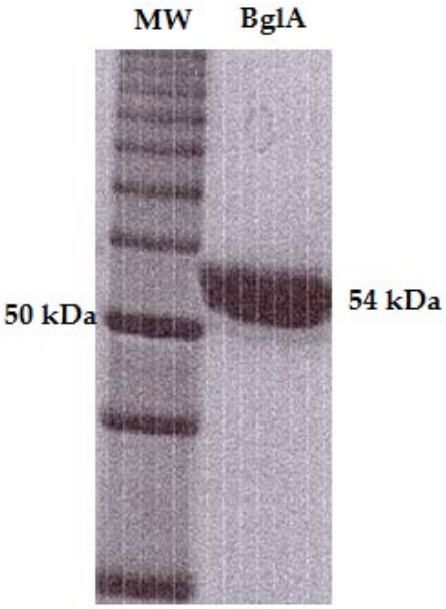

Figure 4: Purification of the BglA polypeptide as a glutathione S-transferase fusion protein. The 54-kDa polypeptide, corresponds to the purified recombinant BglA after cleavage with PreScission™ protease. The molecular mass standard (MW) is the 10-kDa protein ladder from Gibco BRL.

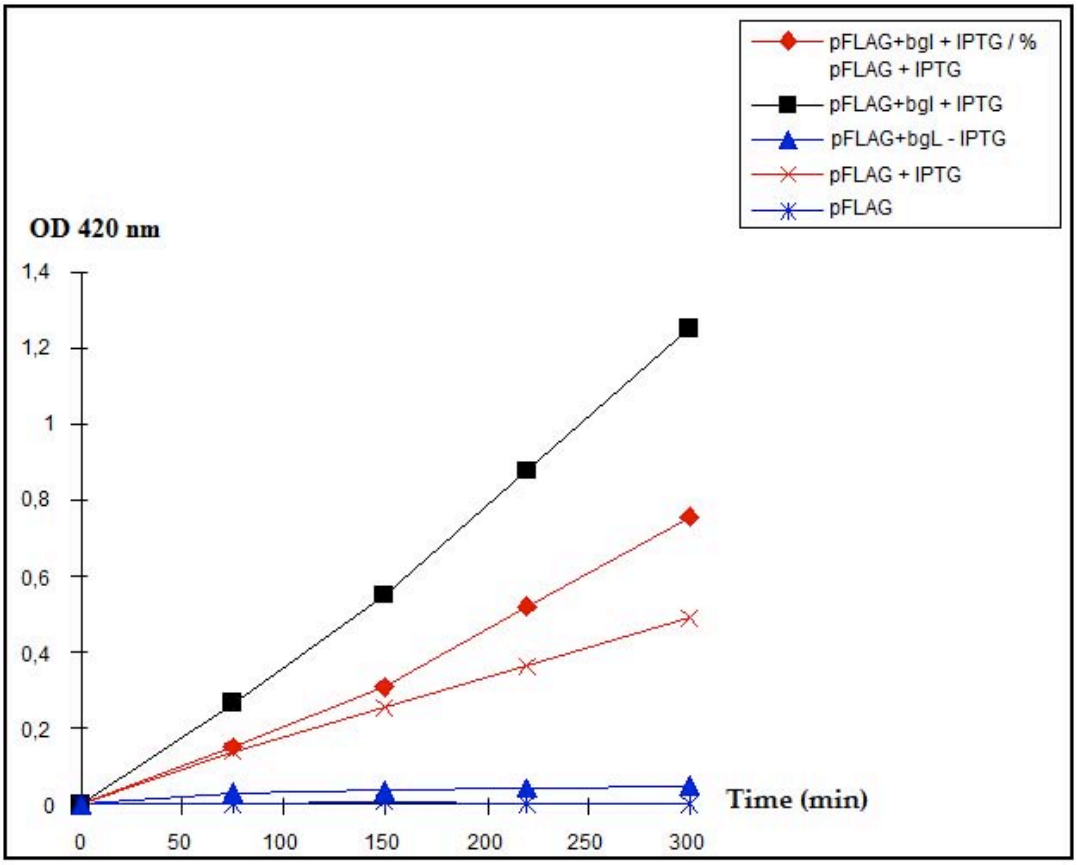

Figure 5: Detection of enzymatic activity of recombinant BglA polypeptide on o-nitrophenyl-β-D-galactopyranoside (ONPG). The chromogenic substrate ONPG was used in ascending concentrations in steps of 0.2 mg/ml in Z-buffer (see Materials and Methods). Recombinant BglA polypeptide was added to the solution with the final concentration of 40 ng/µl. Hydrolysis of ONPG to o-nitrophenol and β-D-galactose was determined in a photospectrometre at 420 nm.