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Differential Regulation of *Listeria monocytogenes* Internalin and Internalin-Like Genes by σ^B and PrfA as Revealed by Subgenomic Microarray Analyses

Patrick McGann,[1] Sarita Raengpradub,[1] Renata Ivanek,[2] Martin Wiedmann,[1] and Kathryn J. Boor[1]

Abstract

The *Listeria monocytogenes* genome contains more than 20 genes that encode cell surface–associated internalins. To determine the contributions of the alternative sigma factor σ^B and the virulence gene regulator PrfA to internalin gene expression, a subgenomic microarray was designed to contain two probes for each of 24 internalin-like genes identified in the *L. monocytogenes* 10403S genome. Competitive microarray hybridization was performed on RNA extracted from (i) the 10403S parent strain and an isogenic Δ*sigB* strain; (ii) 10403S and an isogenic Δ*prfA* strain; (iii) a (G155S) 10403S derivative that expresses the constitutively active PrfA (PrfA*) and the Δ*prfA* strain; and (iv) 10403S and an isogenic Δ*sigB*Δ*prfA* strain. σ^B- and PrfA-dependent transcription of selected genes was further confirmed by quantitative reverse-transcriptase polymerase chain reaction. For the 24 internalin-like genes examined, (i) both σ^B and PrfA contributed to transcription of *inlA* and *inlB*, (ii) only σ^B contributed to transcription of *inlC2, inlD, lmo0331*, and *lmo0610*; (iii) only PrfA contributed to transcription of *inlC* and *lmo2445*; and (iv) neither σ^B nor PrfA contributed to transcription of the remaining 16 internalin-like genes under the conditions tested.

Introduction

Listeria monocytogenes is a gram-positive, facultative intracellular pathogen responsible for severe foodborne infections in mammals and a variety of other vertebrates (reviewed in Low and Donachie, 1997; Vazquez-Boland *et al.*, 2001). Internalin proteins play a critical role in the ability of this pathogen to promote its internalization into a number of different nonphagocytic mammalian cells (Cabanes *et al.*, 2002; Bonazzi and Cossart, 2006). *L. monocytogenes* strains differ in the number of internalin genes encoded in their genomes; the four strains sequenced to date have between 24 and 29 internalin genes (Nelson *et al.*, 2004). A total of 11 internalins (InlA, InlB, InlC, InlC2, InlD, InlE, InlF, InlG, InlH, InlI, and InlJ) have been characterized through phenotypic analyses of strains bearing appropriate null mutations. Of these, null mutations in four internalin genes (*inlA, inlB, inlC*, and *inlJ*) resulted in reduced invasion or virulence in tissue culture or animal models (Engelbrecht *et al.*, 1996; Dramsi *et al.*, 1997; Raffelsbauer *et al.*, 1998; Sabet *et al.*, 2005). While the specific functions of the majority of *L. monocytogenes* internalins are currently undefined, this family of proteins appears to have diverse roles in the physiology and virulence of this pathogen (Popowska and Markiewicz, 2006).

Many *L. monocytogenes* genes involved in virulence are at least partially controlled by the pleiotropic transcriptional activator PrfA

[1]Department of Food Science and [2]Department of Population Medicine and Diagnostic Sciences, Cornell University, Ithaca, New York.

(Chakraborty *et al.*, 1992). Among the internalins, both InlA and InlB are partially controlled by PrfA (Lingnau *et al.*, 1995), while the *inlC* gene contains overlapping PrfA-dependent and -independent promoters (Luo *et al.*, 2004). The stress responsive alternative sigma factor σ^B also regulates transcription of a number of genes with confirmed or suspected roles in *L. monocytogenes* virulence (Kazmierczak *et al.*, 2003, 2006; Garner *et al.*, 2006), including, but not limited to, internalin genes such as *inlA* and *inlB* (Kim *et al.*, 2004; Kazmierczak *et al.*, 2006; McGann *et al.*, 2007b). Mounting evidence supports the existence of an adaptive transcriptional regulatory network between *L. monocytogenes* PrfA and σ^B that includes σ^B-dependent regulation of *prfA* transcription as well as coregulation of selected virulence genes (e.g., *bsh*) by both PrfA and σ^B (Chakraborty *et al.*, 1992; Bohne *et al.*, 1996; Wiedmann *et al.*, 1998; Nadon *et al.*, 2002; Kazmierczak *et al.*, 2003, 2006; Kim *et al.*, 2005; Rauch *et al.*, 2005; Gray *et al.*, 2006). Interestingly, as a Δ*sigB* null mutant is attenuated after intragastric, but not after intravenous, infection of guinea pigs (Garner *et al.*, 2006), σ^B appears to be particularly important for regulating transcription during the gastrointestinal stages of *L. monocytogenes* infection, while PrfA appears to be more important during the systemic and intracellular stages of infection (Kazmierczak *et al.*, 2006). We thus hypothesized that identification and characterization of *L. monocytogenes* internalin-like genes as PrfA- and/or σ^B-dependent could provide insight into whether specific internalins are likely to be expressed during gastrointestinal or systemic stages of infection, thus contributing to our understanding of the various, and still undefined, roles of the different *L. monocytogenes* internalins.

To determine relative contributions of σ^B and PrfA to internalin gene expression, we used a subgenomic microarray containing the 24 internalin-like genes identified in *L. monocytogenes* strain 10403S, including those encoding the 11 currently characterized internalins. Transcriptional contributions of σ^B and PrfA were also confirmed by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) for selected genes.

Materials and Methods

Bacterial strains and growth

L. monocytogenes 10403S (serotype 1/2a [Bishop and Hinrichs, 1987]) and three isogenic, nonpolar null mutants, including Δ*sigB* (FSL A1-254 [Wiedmann *et al.*, 1998]), Δ*prfA* (FSL B2-046 [Wong and Freitag, 2004]), and Δ*sigB*Δ*prfA* (FSL B2-068 [McGann *et al.*, 2007b]) were used in this study. Strain NF-L753, an otherwise isogenic 10403S strain that contains the (G155S) *prfA** allele, which encodes a constitutively active PrfA protein referred to as PrfA*, was kindly provided by Nancy Freitag (University of Washington, Seattle).

To enhance the relative proportion of cells in log phase, for all experiments, each strain was grown in brain heart infusion (BHI) broth at 37°C with shaking (200 rpm) to $OD_{600} = 0.4$, then diluted 1:100 into fresh BHI and grown to $OD_{600} = 0.4$. Cells were then exposed to conditions previously reported to activate PrfA or σ^B (0.2% charcoal or 0.3 M NaCl, respectively [Ripio *et al.*, 1996; Sue *et al.*, 2003]). Specifically, to collect RNA for identification of PrfA-dependent genes, 0.2% charcoal was added to early-log phase ($OD_{600} = 0.4$) 10403S and Δ*prfA* cells, which were subsequently incubated with shaking for 120 minutes at 37°C. To collect RNA for identification of PrfA- and σ^B-dependent genes, early-log phase 10403S and Δ*sigB*Δ*prfA* cells were incubated with shaking for 120 minutes at 37°C in BHI with 0.2% charcoal with NaCl added (0.3 M final concentration) for the final 10 minutes. To collect RNA for identification of σ^B-dependent genes, early-log phase 10403S and Δ*sigB* cells were incubated for 120 minutes in BHI with NaCl added (0.3 M final concentration) for the final 10 minutes. To collect RNA for identification of PrfA-dependent genes in the *prfA** strain, early-log phase *prfA** and Δ*prfA* cells were subsequently incubated for 120 minutes in BHI. Following each treatment, RNA was stabilized by the addition of two volumes of RNAprotect™ (Qiagen, Valencia, CA). Bacterial cells were then harvested by centrifugation and stored at −80°C for no longer than 24 hours before RNA isolation. A total of three independent replicates were completed on three different days for each experiment.

RNA collection and purification

Total RNA was extracted as previously described (Kazmierczak *et al.*, 2006) except that contaminating DNA was removed using Turbo DNase according to the manufacturer's instructions (Ambion Inc., Austin, TX). Purified RNA was precipitated and stored at −80°C.

Identification of internalin and internalin-like genes in L. monocytogenes *10403S*

A total of 11 internalin genes were previously identified in the *L. monocytogenes* 10403S genome (*inlA, inlB, inlC, inlC2, inlD, inlE, inlF*, and *inlG* [Tsai *et al.*, 2006] and *lmo0171, lmo0801*, and *lmo2026* [S. Milillo and M. Wiedmann, unpublished results]). To determine if additional internalin genes might be present in the 10403S genome, we designed primers (see Suppl. Table S1; all supplementary materials are available at www.liebertpub.com/fpd) for the 15 internalin-like genes that are present in *L. monocytogenes* EGD-e (Nelson *et al.*, 2004), but that had not previously been reported in 10403S. We reasoned that the EGD-e genome was an appropriate template as both EGD-e and 10403S are serotype 1/2a (Glaser *et al.*, 2001; Zhang *et al.*, 2003) and both belong to the *L. monocytogenes* phylogenetic lineage II (Piffaretti *et al.*, 1989; Zhang *et al.*, 2003). While 13 of the 15 targeted internalin genes were successfully amplified in strain 10403S (see Suppl. Table S1), no PCR products were obtained with the primers for *lmo0549* and *lmo1289*. PCR for *lmo0549* and *lmo1289* was repeated with three additional primer sets designed to target different regions of both genes, but no products were generated with any of the four primer sets. Therefore, PCR products from 13 internalin genes were sequenced to enable design of the 70-mer oligonucleotide probes for use in the microarray; PCR product purification and sequencing was performed using standard procedures previously described by our group (Tsai *et al.*, 2006).

When the genome sequence for *L. monocytogenes* 10403S (Anonymous, 2007), the parent strain used here, became available (after completion of the experiments described here), nucleotide and Protein BLAST searches using the internalin and internalin-like gene sequences from EGD-e (Glaser *et al.*, 2001), as well as the sequences for the seven internalin genes found in *L. monocytogenes* F2365 and F6854 (Nelson *et al.*, 2004) were performed as an additional strategy to identify internalin-like genes in 10403S. None of the internalin genes unique to F2365 and F6854 were identified in 10403S. While the BLAST searches confirmed the absence of *lmo1289* from the 10403S genome (as predicted by our PCR results), a 10403S gene with ~75% identity with EGD-e *lmo0549* was identified. The genome of 10403S was further examined for the presence of leucine-rich repeats, a feature conserved among currently recognized internalin proteins (Kobe and Deisenhofer, 1995). This approach confirmed the existence of the 25 internalins and internalin-like genes identified through the strategies described above; no additional, novel internalin-like genes were identified. In summary, *L. monocytogenes* 10403S contains 25 internalin and internalin-like genes, including *inlC2* and *inlD* rather than *inlH*, which is present in EGD-e, and *lmo0549*. Consequently, with the exception of *lmo0549,* the microarray used in the study described here is an appropriate tool for monitoring transcript levels for the 24 remaining internalin and internalin-like genes present in 10403S.

Design and validation of 70-mer oligonucleotides for microarray

ArrayOligoSelector (http://arrayoligosel.sourceforge.net/) was used to design two 70-mer oligonucleotides for each target gene included in the subgenomic microarray (see Suppl. Table S2, which also shows the primers positions in each gene). A concatenated sequence containing the 24 internalin genes determined to be present in the 10403S genome (including the *inlC2* and *inlD* sequences from strain 10430S and excluding *inlH*, which is present in EGD-e, but not in 10403S), 25 housekeeping genes, and four control genes (*prfA, sigB*, the PrfA-dependent *plcA*, and the σ^B-dependent *opuCA*) was used as input for ArrayOligoSelector. As both PrfA and σ^B are auto-regulated (Mengaud *et al.*, 1991; Becker *et al.*, 1998), the genes encoding each protein (i.e., *prfA* and *sigB*) as well as genes that are regulated by each protein (i.e., *plcA* and

opuCA) were included to allowed us to determine if increased transcript levels for genes regulated by each protein occurred in parallel with (i) increased transcription of the genes encoding the regulators; (ii) increased activity of regulators; or (iii) both. To ensure homology between each oligonucleotide and the targeted *L. monocytogenes* 10403S sequence, a region comprising 100 nt upstream and downstream of each 70-mer oligonucleotide was sequenced; all oligonucleotides showed greater than 95% homology with the *L. monocytogenes* 10403S sequences (Suppl. Table S2).

Microarray preparation and printing

Microarray preparation and printing were performed as described by McGann *et al.* (2007a). Briefly, oligonucleotides (Suppl. Table S2) were synthesized by Operon Biotechnologies Inc. (Huntsville, AL). Prior to use, oligonucleotides were suspended in $3 \times$ SSC to a final concentration of 30 mM. To assist in background correction, 70-mer oligonucleotides targeting five yeast genes encoding the mating pheromone α-factors (*mfα1, mfα2*), mating-type α-factor pheromone receptor (*ste3*), actin (*act1*), and a GTP-binding protein involved in the regulation of the cyclic AMP pathway (*ras1*), were used for negative controls (Wu *et al.*, 2001). To assist in signal normalization, serial dilutions of 10403S chromosomal DNA ranging in concentration from 200 ng/μL to 0.78125 ng/μL were prepared in printing buffer and spotted. All oligonucleotides and controls were spotted in duplicate on Corning UltraGAPS slides (Corning, NY) using a custom built XYZ microarrayer.

Probe labeling and microarray hybridization

Probe labeling and microarray hybridization were performed as described by McGann *et al.* (2007a). Briefly, cDNA was synthesized from 10 μg purified total RNA and labeled with Alexa Fluor® dyes using the SuperScript™ Plus Indirect cDNA labeling system (Invitrogen Inc., Carlsbad, CA). For the strains to be compared, labeled cDNA was resuspended in 50 μL of hybridization buffer (5×SSC, 0.1% SDS, 0.1mM dithiothreitol (DTT), 0.5×formamide, 600 μg/mL salmon sperm DNA), denatured at 95°C for 5 minutes, then applied to the slides using mSeries LifterSlips™ (Erie Scientific, Portsmouth, NH). Hybridization was conducted in a water bath at 42°C overnight. The slides were dried by centrifugation and scanned using a GenePix 4000b scanner (Axon Instruments Inc., Foster City, CA). Acquired images were analyzed using GenePix Pro 6.0 (Molecular Devices Corp., Sunnyvale, CA).

Microarray data analysis

Analyses of microarray data were performed in R (http://www.R-project.org) with Bioconductor (Gentleman *et al.*, 2004) using LIMMA software (Smyth, 2005) as described by McGann *et al.* (2007a). For normalization within arrays, the data were weighted for the housekeeping genes and genomic DNA controls and normalized using print-tip loess (Smyth and Speed, 2003). Finally, between-array normalization was performed by application of scale-normalization to scale the log-ratios to the same median-absolute-deviation across arrays.

Due to the small number of targeted genes, two complete arrays were printed on each microarray slide. As two different probes, printed in duplicate, were used for each gene on a single array, the microarray design generated eight replicate spots for each gene per slide. Expression profiles were measured from three independent RNA extractions, resulting in 24 replicate spots per gene (12 from each probe). To provide the most comprehensive and robust analyses of these data, results from the two independent probes for each gene were analyzed and reported separately. In addition, the two arrays on each slide were treated as independent blocks for statistical examination and analyzed accordingly. A linear model was fitted to the normalized log ratios and four B-statistics, t-statistics, and p values were generated for each gene (resulting in four separate expression profiles with corresponding p values for each gene representing two different 70-mer microarray probes in each of two separate array blocks on the same slide). Raw data and microarray files in MIAME format are available through the Gene Expression Omnibus (GEO) (Edgar *et al.*, 2002; Barrett *et al.*, 2005) with accession number GSE6471.

Quantitative RT-PCR using TaqMan

All TaqMan primers and probes (Suppl. Table S3) were designed using Primer Express software (Applied Biosystems, Foster City, CA). Probes with MGB quencher dye were synthesized by Applied Biosystems and probes with QSY7 quencher dye were synthesized by MegaBases Inc. (Evanston, IL). Quantitative RT-PCR was performed in 25 μL reactions as previously described (Kazmierczak *et al.*, 2006) using iTaq Supermix with Rox (BioRad, Hercules, CA). All qRT-PCR experiments were performed in triplicate from the same three RNA isolations used to perform microarray analyses. Absolute transcript levels were normalized to the geometric mean of the two housekeeping genes *rpoB* and *gap* as described previously (Chaturongakul and Boor, 2006; Kazmierczak *et al.*, 2006); all data were log transformed to achieve a normal distribution. To compare transcript levels from two strains or conditions, standard two-sample *t* tests were employed. To compare transcript levels from more than two strains or conditions, a one-way ANOVA with Tukey's multiple comparison procedure was used for data analysis. All statistical analyses were performed in S-Plus 6.2 (Insightful Corp, Seattle, WA).

Results

Competitive hybridization using a subgenomic microarray targeting 24 internalin-like genes present in *L. monocytogenes* 10403S as well as (i) *prfA*, (ii) the PrfA-dependent gene *plcA*, (iii) *sigB*, and (iv) the σ^B-dependent gene *opuCA*, was performed on RNA extracted from 10403S and three otherwise isogenic strains (Δ*sigB*, Δ*prfA*, and Δ*sigB*Δ*prfA*) grown under conditions reported to maximize expression of the active state of PrfA, σ^B, or both (Ripio *et al.*, 1996; Sue *et al.*, 2003). As no differences in transcript levels for any internalin genes were found between 10403S and the Δ*prfA* strain under conditions reported to maximize PrfA activity (growth in BHI with activated charcoal; Ripio *et al.*, 1996), competitive hybridization was also performed with the Δ*prfA* strain and a 10403S strain with the *prfA** (G155S) allele, which expresses the constitutively active PrfA* protein (Shetron-Rama *et al.*, 2003). Therefore, in total, four different competitive microarray hybridizations were performed, including (i) 10403S and Δ*sigB*, (ii) 10403S and Δ*prfA*, (iii) 10403S *prfA** and Δ*prfA*, and (iv) 10403S and Δ*sigB*Δ*prfA*. σ^B- and PrfA-dependent expression of selected genes was also confirmed by quantitative RT-PCR.

Validation of microarrays by qRT-PCR

Initial examination of the microarray expression profiles indicated that 21 genes, including 18 internalin-like genes, were differentially regulated by σ^B and/or PrfA (Table 1), as supported by significantly higher transcript levels in the parent strain for at least one probe in at least one of the four strain comparisons. Ten genes, however, only showed significantly higher transcript levels in one probe in a given strain comparison (e.g., *lmo0327* in the 10403S–Δ*sigB* comparison; Table 1) or only for the probe(s) in one of the two blocks printed on a given glass slide (e.g., *lmo0262* in the 10403S–Δ*sigB* comparison; Table 1). Overall, eight internalin genes and three control genes (*plcA, prfA*, and *opuCA*) showed significant differential regulation for both probes and for both array blocks in at least one of the four comparisons (Table 1, genes marked with **). To confirm the microarray data, we performed qRT-PCR to validate differential transcription of (i) two genes (*lmo0331* and *lmo0610*) that showed significant differences for both probes and in both blocks in each microarray comparison with a Δ*sigB* strain; (ii) a total of eight genes that showed inconsistent evidence for differential expression in the 10403S–Δ*sigB* (*lmo0327*), the 10403S–Δ*sigB*Δ*prfA* (*lmo0801, lmo1290, lmo2027*), or both (*lmo0514, lmo0732, lmo2026, lmo2396*) comparisons, (iii) one control gene (*lmo2470*) that did not show evidence of differential expression in any microarray comparison; (iv) and *lmo2445*, which showed a consistently significant, but $\leq$1.5-fold, difference in transcript levels in the 10403S–Δ*sigB* microarray experiments. Quantitative RT-PCR on the eight selected genes that showed inconsistent evidence of differential expression (*lmo0327, lmo0514, lmo0732, lmo0801, lmo1290, lmo2026, lmo2027, lmo2396*) found no significant differences in transcript levels for these genes between the wildtype and the appropriate mutant (Δ*sigB*Δ*prfA* or Δ*sigB*; Suppl. Table S4).

Table 1. Summary of Microarray Results

Gene locus (common name)[a]	Differences in transcript levels in comparisons between[b]							
	10403S/ΔsigB		*10403S/ΔprfA*		*prfA*/ΔprfA*		*10403S/ΔsigBΔprfA*	
	Probe 1	*Probe 2*	*Probe 1*	*Probe 2*	*Probe 1*	*Probe 2*	*Probe 1*	*Probe 2*
lmo0171	—	—	—/—	—/—	—/—	—/—	—/—	—/—
*lmo0262 (inlG)**	3/—	2.6/—	—/—	—/—	—/—	—/—	2.8/—	—/—
(*inlC2*)**	60/52	50/47	—/—	—/—	—/—	—/—	63/58	49/38
(*inlD*)**	12/10	18/19	—/—	—/—	—/—	—/—	22/18	16/15
*lmo0264 (inlE)**	2/—	—/—	—/—	—/—	—/—	—/—	—/—	—/—
*lmo0327**	2.5/2.4	—/—	—/—	—/—	—/—	—/—	—/—	—/—
*lmo0331***	2.7/2.5	3.3/3.2	—/—	—/—	—/—	—/—	2.4/3	2.7/3.1
lmo0333 (inlI)	—/—	—/—	—/—	—/—	—/—	—/—	—/—	—/—
lmo0409 (inlF)	—/—	—/—	—/—	—/—	—/—	—/—	—/—	—/—
*lmo0433 (inlA)***	42/44	40/46	—/—	—/—	2.2/2.3	2.1/2.6	86/111	114/105
*lmo0434 (inlB)***	39/38	21/20	—/—	—/—	2.1/2.4	3.2/2.9	108/120	91/92
*lmo0514**	1.6/1.5	1.4/—	—/—	—/—	—/—	—/—	4.6/4.4	—/—
*lmo0610***	27/24	20/21	—/—	—/—	—/—	—/—	29/22	31/28
*lmo0732**	2/—	—/—	—/—	—/—	—/—	—/—	—/—	3.3/—
*lmo0801**	—/—	—/—	—/—	—/—	—/—	—/—	2/—	—/—
lmo1136	—/—	—/—	—/—	—/—	—/—	—/—	—/—	—/—
*lmo1290**	—/—	—/—	—/—	—/—	—/—	—/—	2.9/—	—/—
*lmo1786 (inlC)***	—/—	—/—	—/—	—/—	2.4/2.4	2.6/2.5	—/—	—/—
*lmo2026**	1.9/1.3	—/—	—/—	—/—	—/—	—/—	—/—	2.5/4
*lmo2027**	—/—	—/—	—/—	—/—	—/—	—/—	2.1/—	—/—
*lmo2396**	2.2/—	—/—	—/—	—/—	—/—	—/—	2.9/—	—/—
*lmo2445***	1.4/1.3	1.5/1.4	—/—	—/—	2.8/3.1	2.4/2.5	—/—	—/—
lmo2470	—/—	—/—	—/—	—/—	—/—	—/—	—/—	—/—
lmo2821 (inlJ)	—/—	—/—	—/—	—/—	—/—	—/—	—/—	—/—
*lmo0201 (plcA)***	—/—	—/—	13/12	11/10	53/55	47/49	8.1/8.3	10.5/9.3
*lmo0200 (prfA)***	2.6/2.4	1.9/2	NA	NA	NA	NA	NA	NA
*lmo1428 (opuCA)***	24/23	34/34	—/—	—/—	—/—	—/—	33/35	35/33
lmo0895 (sigB)	NA	NA	—/—	—/—	—/—	—/—	NA	NA

[a]Gene locus and common name are based on the annotation of the *Listeria monocytogenes* EGD-e genome available at NCBI (http://www.ncbi.nlm.nih.gov), accession number AL591824. A single asterisk * indicates genes showing a significant difference in spot intensities in just one of the two probes and/or one of the two array blocks. A double asterisk ** indicates genes showing a significant difference in spot intensities with both probes and array blocks.

[b]Differences in transcript levels expressed as fold differences were calculated using Bioconductor for R (http://www.bioconductor.org/biocLite.R) based on the microarray data, and equal the average spot intensity for the first strain listed (10403S parent strain or *prfA**) divided by the average spot intensity for the second strain listed (i.e., the mutant strain). Each probe, which was reported separately, consists of two expression values corresponding to the average parent strain spot intensity divided by the average mutant spot intensity from the upper and lower arrays from three separate slides from each of three replicate RNA isolations. For example, the value 60/52 indicates a 60-fold and a 52-fold change in spot intensities for the gene between the parent and mutant strain from the upper and lower array, respectively (See *Methods* for further details). Dashes (—) indicate no significant difference in spot intensities. NA indicates not applicable (since the target gene for the probe was absent from the mutant strain).

Transcript levels for *inlG* and *inlE*, the other two genes that showed inconsistent differential expression in this study, were reported not to differ between the wildtype and Δ*sigB* strains by qRT-PCR analysis (McGann *et al.*, 2007b). Quantitative RT-PCR also showed that differences in transcript levels were not significant for the one gene (i.e., *lmo2445*) that showed consistently significant differential expression in the 10403S–Δ*sigB* microarray comparison (i.e., significant differences in both probes and both blocks), although at ratios $\leq$1.5. Thus, a given gene was only considered to be differentially expressed if (i) both probes and both blocks showed significant differences and (ii) transcript ratios were >2.0, consistent with previous reports by others (Milohanic *et al.*, 2003; Chatterjee *et al.*, 2006).

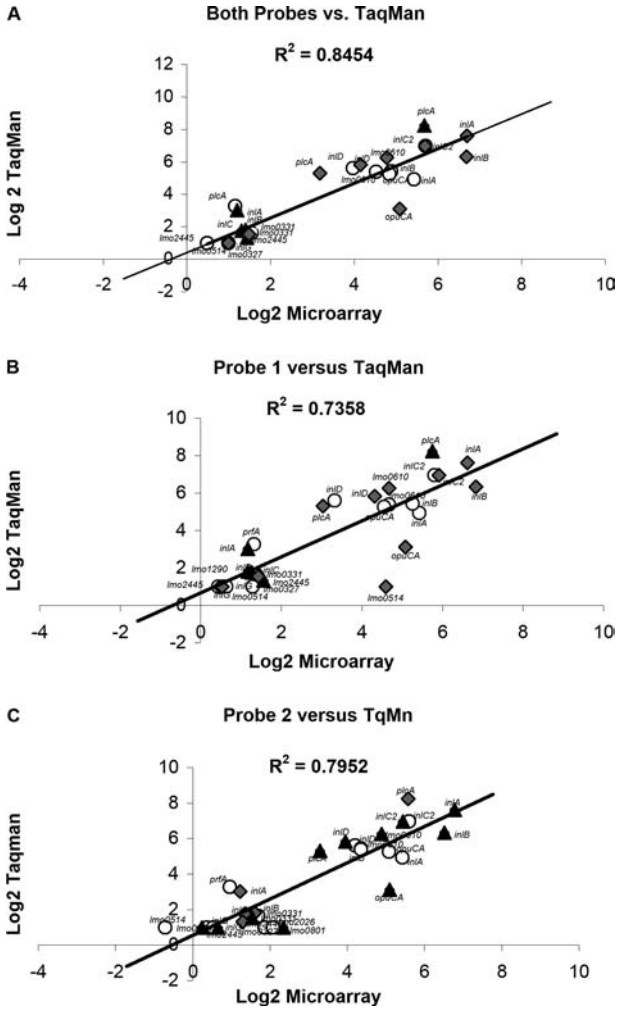

FIG. 1. Correlation between quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) (TaqMan) results and microarray results based on (**A**) the average of both probes, (**B**) probe 1 only, and (**C**) probe 2 only. Fold changes in expression in the 10403S parent strain relative to the 10403S Δ*sigB* strain (○), the PrfA* strain relative to the 10403SΔ*prfA* strain (♦), and the 10403S parent strain relative to the 10403SΔ*sigB*Δ*prfA* strain (▲) were log transformed and plotted against each other to evaluate correlations. Quantitative RT-PCR data for *inlA, inlB, inlC, inlC2, inlD, inlE, inlG*, and *prfA* transcript levels for the 10403S–Δ*sigB* comparison as well as *inlA, inlB, inlC2, inlD, inlG*, and *plcA* transcript levels for the 10403S–Δ*sigB*Δ*prfA* comparison have been reported elsewhere (McGann *et al.*, 2007b).

To further evaluate correlations between qRT-PCR and microarray data, log transformed fold differences based on qRT-PCR and based on microarray data for probe 1 only, probe 2 only, and both probes were each plotted against each other (Fig. 1); qRT-PCR data for *inlA, inlB, inlC, inlC2, inlD, inlE, inlG*, and *prfA* transcript levels for the 10403S–Δ*sigB* comparison as well as for *inlA, inlB, inlC2, inlD, inlG*, and *plcA* transcript levels for the 10403S–Δ*sigB*Δ*prfA* comparison have been reported elsewhere (McGann *et al.*, 2007b). If both methods yield identical results, the slope from the comparisons would equal 1 when the data are plotted against each other, as in Fig. 1. A higher Pearson correlation coefficient (R^2) was achieved if analyses were conducted using the average value obtained from both microarray probes for a given gene (Fig. 1A; $R^2 = 0.8454$) rather than just probe 1 (Fig. 1B; $R^2 = 0.7358$) or probe 2 (Fig. 1C; $R^2 = 0.7952$), indicating that the use of two probes per gene rather than one, results in a better linear correlation between qRT-PCR and microarray analyses.

σ^B-dependent transcription of internalin-like genes in L. monocytogenes

As exposure of log-phase *L. monocytogenes* to 0.3 M NaCl in BHI for 10 minutes results in high levels of σ^B activity (Sue *et al.*, 2003), competitive hybridization was performed with RNA isolated from 10403S and the Δ*sigB* strain cultured under these conditions. Analyses of the microarray expression profiles revealed that six internalin genes (*inlA, inlB, inlC2, inlD, lmo0331*, and *lmo0610*), and the σ^B-dependent gene *opuCA* had significantly higher transcript levels in the parent strain as compared to the Δ*sigB* strain in both probes and in both blocks (Table 2). Differences in transcript levels

Table 2. Internalin and Internalin-Like Genes Showing Significant Changes in Transcript Levels

	Differences in transcript levels for comparisons between[b]							
	10403S/ΔsigB		*10403S/ΔprfA*		*PrfA*/ΔprfA*		*10403S/ΔsigBΔprfA*	
Gene locus (common name)[a]	*Microarray*	*TaqMan*	*Microarray*	*TaqMan*	*Microarray*	*TaqMan*	*Microarray*	*TaqMan*
inlC2	52	125*	NS	NS	NS	—	52	125*
InlD	15	49*	NS	NS	NS	—	18	57*
lmo0331	2.9	3	NS	—	NS	NS	3.1	2.9
lmo0433 (*inlA*)	43	30*	NS	NS	2.3	8.1	104	197*
lmo0434 (*inlB*)	29	43*	NS	NS	2.7	3.5	103	80*
lmo0610	23	42	NS	NA	NS	—	28	76
lmo1786 (inlC)	NS	NS	NS	NS	2.5	3.4	NS	NS
lmo2445	NS	—	NS	—	2.7	2.5	NS	NS
opuCA	29	38*	NS	NS	NS	NS	34	40*
plcA	NS	NS	11.4	73*	51	302	9	9
prfA	2.2	NS	NA	NA	NA	NA	NA	NA

[a]Gene locus and common name are based on the annotation of the *Listeria monocytogenes* EGD-e genome available at NCBI (http://www.ncbi.nlm.nih.gov), accession number AL591824.

[b]Differences in transcript levels are expressed as fold differences between the first strain listed (10403S parent strain or *prfA**) and the second strain listed (i.e., the mutant strain). Differences in transcript levels based on the microarray data were calculated using Bioconductor for R (http://www.bioconductor.org/biocLite.R), and equal the average spot intensity for the first strain listed (10403S parent strain or *prfA**) divided by the average spot intensity for the second strain listed (i.e., the mutant strain) from each of three replicate RNA isolations spotted on three separate slides containing two arrays with two probes per gene per array (see *Methods* for details). Differences in transcript levels based on TaqMan data represent the absolute difference in mRNA transcript levels (normalized to the geometric mean of *rpoB* and *gap* transcript levels) between the first strain listed (10403S parent strain or *prfA**) and the second strain listed (i.e., the mutant strain). Fold changes are only presented where statistically significant using an α value of 0.05. Values marked with an asterisk (*) were reported elsewhere (McGann *et al.*, 2007b). NS indicates no significant difference in mRNA transcript levels; dashes (—) indicate quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was not performed; NA indicates not applicable (since the target gene for the probe was absent from the mutant strain).

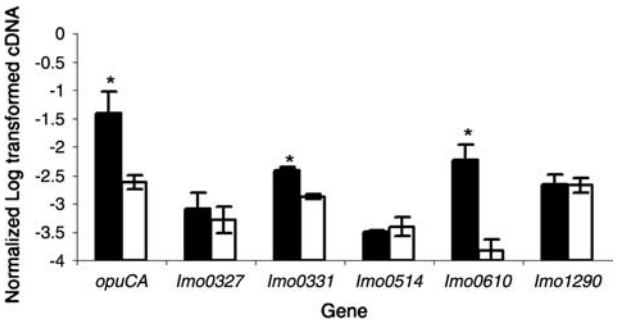

FIG. 2. Transcript levels determined by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) for selected genes identified as differentially expressed between the 10403S parent strain and the Δ*sigB* strain based on one or both probes in the microarray. *lmo1290* was included as a negative control; the microarray did not reveal any differences in transcript levels between 10403S and the Δ*sigB* strain for this gene. Transcript levels were quantified by qRT-PCR and normalized (see *Methods*), and are represented on the y-axis as $\log_{10}$ values. Dark and light bars represent the 10403S parent strain and the Δ*sigB* strain, respectively. Data represent the mean of results from qRT-PCR experiments using three independent RNA isolations; error bars represent one standard deviation. An asterisk indicates that transcript levels for a given gene are significantly different between 10403S and the Δ*sigB* strain.

between 10403S and the Δ*sigB* strain were particularly high (>10-fold) for *inlA, inlB, inlC2, inlD, opuCA*, and *lmo0610*, while *lmo0331* showed less than fourfold higher transcript levels in 10403S (Table 2).

As σ^B-dependent transcription of *inlA, inlB, inlC2*, and *inlD* was previously confirmed by qRT-PCR (Kim *et al.*, 2005; McGann *et al.*, 2007b), qRT-PCR was only used to confirm σ^B-dependent gene transcription of five internalin genes (*lmo0327, lmo0331, lmo0514, lmo0610*, and *lmo1290*) (Fig. 2). For *lmo0331* and *lmo0610*, qRT-PCR showed significantly higher transcript levels in 10403S compared to the Δ*sigB* mutant, consistent with the microarray data, which provided strong evidence for σ^B-dependent transcription of these genes. Consistent with these results, HMM searches (described by Raengpradub *et al.*, 2008) identified putative σ^B promoter sites 55 and 84 nt upstream of *lmo0331* and *lmo0610*, respectively (Fig. 3A). Quantitative RT-PCR found no significant differences in *lmo0327, lmo0514*, and *lmo1290* transcript levels between 10403S and the Δ*sigB* mutant (Fig. 2), consistent with the observation that *lmo0327* and *lmo0514* showed significant differences in transcript levels only with one probe (*lmo0327*) or with less than a twofold change (*lmo0514*).

PrfA-dependent transcription of internalin-like genes in L. monocytogenes

The presence of activated charcoal in growth media was previously reported to result in increased PrfA activity, as evidenced by increased transcription of PrfA-dependent genes (Ripio *et al.*, 1996; Ermolaeva *et al.*, 1999; Milohanic *et al.*, 2003). Therefore, to determine the contributions of PrfA to regulation of internalin gene expression, competitive hybridization was performed on RNA isolated from the 10403S parent and Δ*prfA* strains grown in the presence of activated charcoal. Analyses of the microarray expression profiles revealed that only the PrfA-dependent gene *plcA* showed higher transcript levels in the 10403S parent strain as compared to the Δ*prfA* strain (Table 2), which may be due to the use of a different *L. monocytogenes* strain in our experiments or due to a shorter exposure time to charcoal (2 hours as compared to overnight culture in BHI with charcoal by Milohanic *et al.* [2003]).

As PrfA-dependent transcription of *inlA, inlB*, and *inlC* has been described previously (Lingnau *et al.*, 1995; Engelbrecht *et al.*, 1996; Milohanic *et al.*, 2003; McGann *et al.*, 2007b), we speculated that PrfA activity in *L. monocytogenes* 10403S grown in the presence of activated charcoal was not high enough to induce transcription of these genes to a level where PrfA-dependent transcription would be detectable by the microarray methodology used. Therefore, we used a *L. monocytogenes* 10403S derivative strain carrying the *prfA** (G155S) allele, which expresses the constitutively active PrfA* (Shetron-Rama *et al.*, 2003), for isolation of RNA for microarray experiments. Competitive microarray hybridization using cDNA generated from RNA isolated from the *prfA** and Δ*prfA* strains revealed higher transcript levels for four internalin genes (*inlA, inlB, inlC*, and *lmo2445*) as well as for *plcA* in the *prfA** strain, indicating PrfA-dependent transcription of these genes. Differential expression of *plcA* in the *prfA** strain (as compared to the Δ*prfA* strain) was considerably higher than differential expression of *plcA* between the wildtype *prfA* strain (10403S)

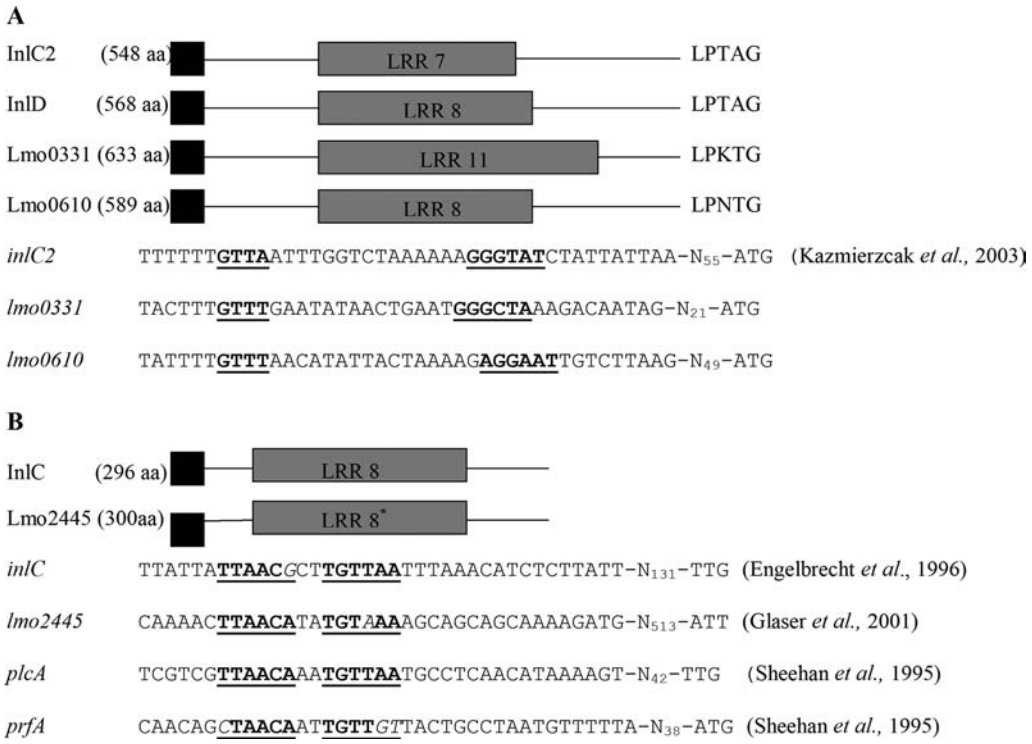

FIG. 3. Representation of (**A**) the structures of the four internalins encoded by σ^B-dependent genes, including the promoter region DNA sequences for these genes, and (**B**) the structures of the two internalins encoded by PrfA-dependent genes, including the promoter region sequences for these genes. The numbers within the gray shaded areas indicate the number of leucine-rich repeat units within each coding region (Raffelsbauer *et al.*, 1998; Hamon *et al.*, 2006). The LPXTG motif for proteins covalently anchored to the cell wall is also indicated. The total number of amino acids in each protein is listed next to the protein name. The DNA sequences corresponding with (**A**) the σ^B promoter sequences and (**B**) the PrfA binding domains (PrfA-box) are underlined and in bold and their distance from each start codon for each reading frame is shown. The PrfA box upstream of *plcA*, which has a perfect palindromic sequence, and upstream of *prfA*, which differs from the perfect palindrome by three mismatches, are shown for comparison. Mismatches are underlined and in italics. No σ^B promoter sequence is given for *inlD* in panel A as the *inlC2D* operon appears to be transcribed from the σ^B promoter upstream of *inlC2*, which is shown here.

and the Δ*prfA* strain grown in BHI with charcoal (50-fold difference in transcript levels as compared to 12-fold, respectively; Table 2), supporting high PrfA activity in the *prfA** strain.

qRT-PCR confirmed that *plcA*, *inlA*, *inlB*, *inlC*, and *lmo2445* transcript levels were significantly higher in the *prfA** strain as compared to the Δ*prfA* strain (Fig. 4; Table 2). These findings are consistent with the presence of PrfA boxes (i.e., 14 bp palindromic sequences with the consensus sequence TTAACAnnTGTTAA (Mengaud *et al.*, 1989; Freitag *et al.*, 1993) upstream of the *inlAB* operon (Dramsi *et al.*, 1993), *inlC* (Engelbrecht *et al.*, 1996), *plcA* (Freitag *et al.*, 1993), and *lmo2445* (Glaser *et al.*, 2001) (Fig. 3B). Quantitative RT-PCR also showed that transcript levels for *lmo2470*, which did not show differential expression in the microarray and which was included as a negative control, did not differ significantly between the *prfA** and the Δ*prfA* strains, confirming that this gene is not PrfA-dependent (Table 1). Quantitative RT-PCR also showed that transcript levels for the PrfA-dependent gene *hly* followed patterns similar to those observed for *plcA*, including higher *hly* transcript levels in the *prfA** strain as compared to the *prfA* wildtype strain grown in charcoal (data not shown).

To further compare PrfA-dependent transcription in the *prfA* wildtype and *prfA** strains grown under different conditions, qRT-PCR data collected here were analyzed together with previously reported qRT-PCR–based transcript levels for *inlA*, *inlB*, *prfA*, *plcA*, and *opuCA* in bacteria exposed to activated charcoal (McGann *et al.*, 2007b), as well as for *prfA*, *plcA*, and *opuCA* in bacteria present in the cytosol and vacuole of infected Caco-2 cells (Kazmierczak *et al.*,

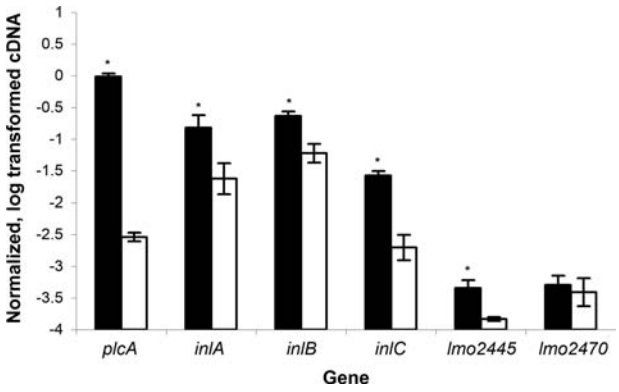

FIG. 4. Transcript levels determined by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) for all genes identified as differentially expressed between the *prfA** (G155S) strain and the Δ*prfA* strain based on one or both probes in the microarray. Dark and light bars represent the *prfA** (G155S) and Δ*prfA* strains, respectively. *lmo2470* was included as a negative control; the microarray did not reveal any differences in transcript levels between the *prfA** (G155S) and the Δ*prfA* strain for this gene. Transcript levels were quantified by qRT-PCR and normalized (see *Methods*), and are represented on the y-axis as $\log_{10}$ values. Data represent the mean of results from qRT-PCR experiments using three independent RNA isolations; error bars represent one standard deviation. An asterisk indicates that transcript levels for a given gene are significantly different between the *prfA** and the Δ*prfA* strains.

2006) (Fig. 5). *prfA* and *plcA* transcript levels in the *prfA** strain (grown in BHI) and the 10403S parent strain present intracellularly in mammalian host cells were similar and significantly higher than *prfA* and *plcA* transcript levels for the wildtype strain grown in BHI with and without charcoal (Fig. 5B), indicating that a *prfA** strain shows *plcA* and *prfA* transcription patterns similar to those in intracellular *L. monocytogenes*. While overall *prfA* transcript levels were similar for the wildtype strain grown in BHI with and without charcoal, *plcA* transcript levels were higher in cells grown in BHI with charcoal, suggesting that charcoal-mediated activation of PrfA sufficient to activate transcription of *plcA*. While *inlA* and *inlB* showed similar transcript levels in both the Δ*prfA* strain and the isogenic parent strain cultured in BHI as well as in the parent strain exposed to activated charcoal, transcript levels for both of these genes were significantly higher in the *prfA** strain grown in BHI (Fig. 5A), consistent with the observation that the PrfA box upstream of *inlAB* is not a perfect palindrome and thus represents a weaker PrfA binding

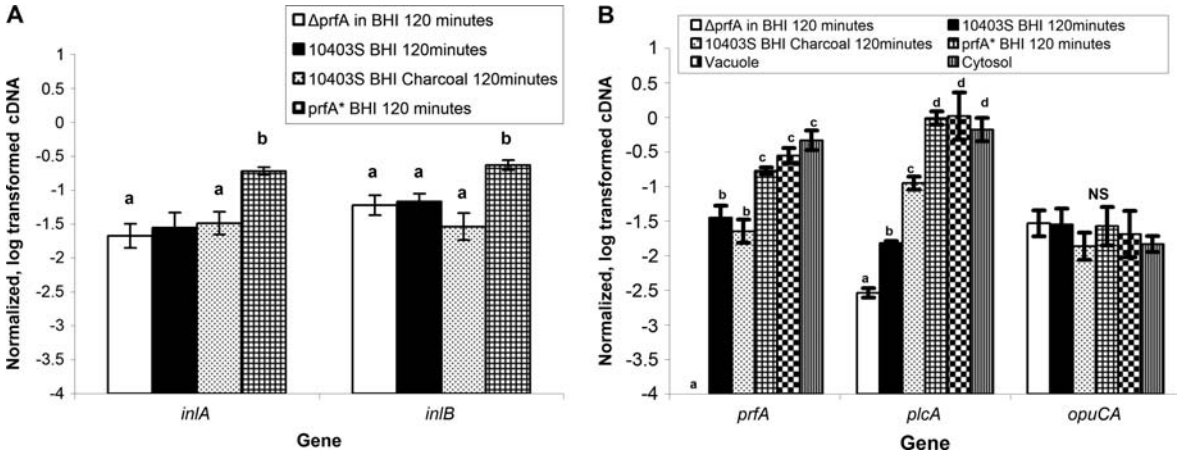

FIG. 5. Transcript levels (based on quantitative reverse-transcriptase polymerase chain reaction [qRT-PCR] data) under different conditions and in different strains (*prfA* wildtype, *prfA**, and Δ*prfA*) for (**A**) *inlA* and *inlB* and (**B**) *prfA*, *plcA*, and *opuCA*. Transcript levels are shown for (i) 10403S *prfA* wildtype in the vacuole and cytoplasm of Caco-2 cells (*prfA*, *plcA*, and *opuCA* only; previously reported by Kazmierczak *et al.*, 2006); (ii) 10403S *prfA* wildtype (cells grown to OD = 0.4 and then exposed to brain heart infusion (BHI) with 0.2% charcoal for 2 hours) (previously reported by McGann *et al.*, 2007b); (iii) 10403S *prfA* wildtype (OD = 0.4 cells grown in BHI for another 2 hours); (iv) Δ*prfA* (OD = 0.4 cells grown in BHI for another 2 hours); and (v) 10403S *prfA** (OD = 0.4 cells grown in BHI for another 2 hours). Transcript levels were quantified using qRT-PCR, normalized (see *Methods*), and represented on the y-axis as $\log_{10}$ values. Data represent the mean of results from qRT-PCR experiments using three independent RNA isolations; error bars represent one standard deviation. Boxes labeled with different letters indicate transcript levels that differed significantly ($p < 0.05$), while boxes labeled with identical letters indicate transcript levels that did not differ significantly (as determined by Tukey's multiple comparison procedure).

site, which is likely to be activated only in the presence of high levels of active PrfA or at a considerable time after initial PrfA activation (Sheehan *et al.*, 1995; Dickneite *et al.*, 1998). We found no significant difference in *opuCA* transcript levels between the Δ*prfA* strain and wildtype strain or *prfA** strain (Fig. 5B), indicating that *opuCA* transcription is not PrfA-dependent, even though a previous report suggested that this gene may be regulated by PrfA (Milohanic *et al.*, 2003).

σ^B and PrfA-dependent transcription of internalin-like genes in L. monocytogenes

As previous reports have suggested that σ^B and PrfA may interact to control expression of some virulence-related genes (Milohanic *et al.*, 2003; Kazmierczak *et al.*, 2006), microarray hybridizations were performed to compare transcript levels for internalin-like genes between the isogenic parent strain and a Δ*sigB*Δ*prfA* strain. Six internalin-like genes (*inlA, inlB, inlC2, inlD, lmo0331*, and *lmo0610*), as well as *plcA* and *opuCA* had higher transcript levels in the 10403S parent strain than in the Δ*sigB*Δ*prfA* strain (Table 1); all of these genes had been identified previously as differentially regulated in either the Δ*sigB* or the Δ*prfA* strain (as compared to the *prfA** strain). *inlA* and *inlB* transcript levels were 110- and 100-fold higher, respectively, in 10403S as compared to the double mutant, substantially higher than the expression differential between the wildtype and Δ*sigB* strain (40- and 30-fold, respectively) or the *prfA** and Δ*prfA* strain (2.3- and 2.6-fold, respectively). The differences in *inlC2, inlD, lmo0331*, and *lmo0610* transcript levels between the parent strain and the Δ*sigB*Δ*prfA* strain were similar to the differences observed between the parent and Δ*sigB* strains (Table 2), further supporting that transcription of these internalin-like genes is σ^B-dependent and PrfA-independent. *inlC* and *lmo2445* transcript levels did not differ significantly between the parent and the Δ*sigB*Δ*prfA* strain, consistent with the microarray-based comparisons of *inlC* and *lmo 2445* transcript levels between the parent and Δ*prfA* strain cultured in BHI with charcoal (Table 1).

qRT-PCR confirmed that *lmo0331* and *lmo0610* transcript levels were higher in the parent strain as compared to the Δ*sigB*Δ*prfA* strain, while *lmo1290* and *lmo2445* transcript levels were similar for both strains, consistent with the microarray data (Fig. 6). Quantitative RT-PCR–based characterization of *inlA, inlB, inlC, inlC2, inlD, opuCA, plcA*, and *opuCA* transcript levels in the parent and Δ*sigB*Δ*prfA* strain have been reported elsewhere (McGann *et al.*, 2007b) and are also consistent with the microarray expression profiles presented here.

Discussion

Microarrays using two 70-mer probes per gene instead of one provide for improved transcriptional profiling accuracy

Microarray technology is a valuable tool for investigating gene expression in both prokaryotic and eukaryotic organisms, however, considerable concern exists about reliability and reproducibility of the data generated (Draghici *et al.*, 2006). For example, cross-hybridization of probes, particularly among probes targeting related genes and gene families, can be of concern, as illustrated by recent studies that showed that a large proportion of microarray probes can

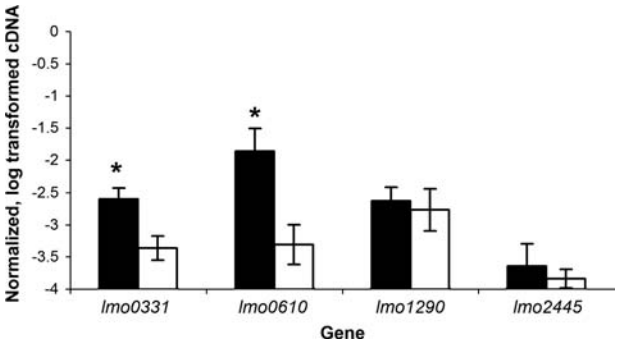

FIG. 6. Transcript levels determined by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) for selected genes identified as differentially expressed between 10403S and the isogenic Δ*sigB*Δ*prfA* strain (based on one or both probes in the microarray; Table 1). *lmo1290* and *lmo2445* were included as negative controls; the microarray did not reveal any differences in transcript levels between 10403S and the Δ*sigB*Δ*prfA* strain for these genes. Transcript levels were quantified by qRT-PCR and normalized (see *Methods*), and represented on the y-axis as $\log_{10}$ values. Dark and light bars represent 10403S and the Δ*sigB*Δ*prfA* strain, respectively. Data represent the mean of results from qRT-PCR experiments using three independent RNA isolations; error bars represent one standard deviation. An asterisk indicates that transcript levels for a given gene are significantly different between 10403S and the Δ*sigB*Δ*prfA* strain.

produce significant cross-hybridization signals (Wu *et al.*, 2005; Zhang *et al.*, 2005). For example, Hughes *et al.* (2001) showed that nontarget sequences with >70% similarity to a 60-mer oligonucleotide probe can cross-hybridize. Various studies have also shown that different oligonucleotide probes for the same gene can yield different signal intensities if they bind to different regions of the gene (Lockhart *et al.*, 1996; Selinger *et al.*, 2000; Hughes *et al.*, 2001). Our data show that the inclusion of two 70-mer probes per gene in a microarray improved the correlation between microarray and qRT-PCR data, with a reduced false discovery rate, as compared to use of a single probe. Use of multiple probes is likely to be particularly critical for transcriptional profiling of gene families that contain genes with highly conserved sequence features, such as the *L. monocytogenes* internalin gene family (Marino *et al.*, 2000), and should provide more accurate and dependable microarray data, as also supported by others (Lockhart *et al.*, 1996; Selinger *et al.*, 2000; Hughes *et al.*, 2001; Relogio *et al.*, 2002). Although validation of microarray expression profiles by other methods such as qRT-PCR has become standard practice (Tan *et al.*, 2003), our findings are consistent with a previous study, which showed that microarray expression profiles may be used without additional experimental verification if multiple gene-specific probes per gene are incorporated, particularly if probes are shorter than 150 nt (Chou *et al.*, 2004).

*A prfA** L. monocytogenes *strain cultured in a standard bacterial medium (i.e., BHI) shows prfA transcript levels and at least some PrfA biological activity similar to that in intracellular bacteria*

To study PrfA-dependent gene expression in *L. monocytogenes*, previous studies have used either *L. monocytogenes* grown in the presence of charcoal (Ripio *et al.*, 1996; Ermolaeva *et al.*, 1999; Milohanic *et al.*, 2003) or *L. monocytogenes* strains with single amino acid substitutions in PrfA that generate constitutively active PrfA proteins. For example, the G155S *prfA** allele encodes a PrfA* protein that appears to be locked in a constitutively active state (Shetron-Rama *et al.*, 2003); consequently a strain with this *prfA** allele exhibits constant, high-level expression of PrfA-dependent genes that are normally induced within the cytosol of infected host cells (e.g., *actA* and *hly*) (Mueller and Freitag, 2005). In this study, we showed that the *L. monocytogenes prfA** (G155S) strain exhibits *prfA, plcA*, and *opuCA* transcript levels similar to those observed in intracellular *L. monocytogenes* isolated from the vacuole and cytosol of infected Caco-2 cells (Kazmierczak *et al.*, 2006), with *prfA* and *plcA* transcript levels that were significantly higher than those observed in a wildtype *prfA* strain cultured with activated charcoal. Thus, use of the *L. monocytogenes prfA** strain appears to represent an appropriate model to study PrfA-dependent gene expression typical for intracellular *L. monocytogenes*.

Our initial microarray experiments showed about 12-fold higher transcript levels for the PrfA-dependent gene *plcA* in the *L. monocytogenes* parent strain as compared to the Δ*prfA* strain when cells were cultured in the presence of activated charcoal for 2 hours. This is consistent with previous reports that showed that *L. monocytogenes* cultured in the presence of activated charcoal display increased expression of key virulence genes, including *prfA, plcA, hly, mpl, actA*, and *plcB* (Ripio *et al.*, 1996; Milohanic *et al.*, 2003; Ermolaeva *et al.*, 2004). However, no significant difference in *inlA, inlB*, and *inlC* transcript levels were found between *L. monocytogenes* 10403S and the isogenic Δ*prfA* strain when cultured in the presence of activated charcoal for 2 hours in our microarray experiments reported here, even though Milohanic *et al.* (2003) reported significantly higher *inlA, inlB*, and *inlC* transcript levels, using a macroarray in an *L. monocytogenes* EGD-e wildtype strain as compared to an EGD-e Δ*prfA* strain (when cultured overnight in the presence of activated charcoal). Using qRT-PCR, our group also did not find significant differences in *inlA, inlB*, and *inlC* transcript levels between a *L. monocytogenes* 10403S parent strain and an isogenic Δ*prfA* mutant cultured in BHI with 0.2% charcoal for 2 hours (McGann *et al.*, 2007b). These data may reflect the fact that some PrfA-dependent genes (e.g., *plcA, hly*) are preceded by a perfect palindromic PrfA binding site, which allows for rapid, high transcriptional activation

of these genes (Camilli *et al.*, 1993; Freitag and Portnoy, 1994; Sheehan *et al.*, 1995), while other virulence genes (e.g., *prfA, mpl, actA, inlA*) are preceded by a weaker PrfA binding site (i.e., an imperfect palindrome with one or more mismatches), which leads to slower and weaker PrfA-dependent transcriptional activation (Sheehan *et al.*, 1995). The presence of PrfA binding sites with different strengths appears to be important for ensuring a hierarchy of virulence gene expression that allows for rapid activation of genes required in the early stages of intracellular infection (e.g., *hly, plcA*), followed by slower, subsequent activation of genes needed later in the intracellular life cycle, e.g., *actA, mpl* (Sheehan *et al.*, 1995). Our results are consistent with this model, as we found differential *plcA* transcription (between the 10403S parent strain and the Δ*prfA* strain) in bacterial cells that were exposed to activated charcoal for 2 hours, while differential expression of PrfA-dependent internalin genes (i.e., *inlAB, inlC, lmo2445*), which are preceded by imperfect palindromes, was only detectable in the *prfA**–Δ*prfA* comparison. Specifically, these genes have palindromic PrfA binding sites that differ from the perfect palindromic PrfA binding sites upstream of *hly* and *plcA* by one (*inlC* and *lmo2445* [Glaser *et al.*, 2001]) or two (*inlAB* [Sheehan *et al.*, 1995; Glaser *et al.*, 2001]) mismatches. These observations suggest that PrfA-dependent transcription of these internalin genes may occur relatively late in the infection cycle (i.e., during intracellular or systemic infection), suggesting the possibility that these gene products contribute to later stages of infection, such as cell-to-cell spread. This hypothesis appears to be consistent with the observation that *inlA* may be important for *L. monocytogenes* crossing of the human maternofetal barrier (Lecuit *et al.*, 2004). Dual regulation of *inlA* by σ^B and PrfA may be required for dual function of this internalin in invasion of intestinal epithelial cells (with activation of transcription during the gastrointestinal stage by σ^B) and crossing of the trophoblastic barrier (with activation of *inlA* transcription during this stage by PrfA). The role of InlA in crossing of the trophoblastic barrier remains unclear, however, as InlA does not appear to contribute to this stage of infection in the guinea pig (Bakardjiev *et al.*, 2004, 2005, 2006).

Internalin genes can be classified based on regulation by σ^B, PrfA, both regulators, or neither

Overall, our data reported here and previously (McGann *et al.*, 2007b) show that σ^B and PrfA contribute to the transcriptional regulation of different internalins such that (i) both σ^B and PrfA contribute to transcription of *inlA* and *inlB*; (ii) only σ^B contributes to transcription of *inlC2, inlD, lmo0331*, and *lmo0610*; and (iii) only PrfA contributes to transcription of *inlC* and *lmo2445*. These findings indicate that *L. monocytogenes* internalin genes are expressed under different environmental conditions, suggesting that differential expression may also occur in different compartments of infected hosts. In particular, coregulation of *inlAB* by σ^B and PrfA may allow appropriate, sensitive control of gene transcription during both gastrointestinal and systemic stages of infection.

All four internalins determined to be σ^B-dependent, but PrfA-independent, (*inlC2, inlD, lmo0331*, and *lmo0610*) are predicted to be covalently attached to the *L. monocytogenes* cell surface through a LPXTG sequence motif (Navarre and Schneewind, 1999). Lmo0610 and Lmo0331 contain 8 and 11 leucine-rich repeat units, respectively, while both *lmo0331* and *lmo0610* encode PKD repeats, which have been proposed to serve as ligand-binding sites in cell-surface proteins (Cabanes *et al.*, 2002). σ^B-dependent regulation of *inlC2, inlD, lmo0331*, and *lmo0610* suggests that these genes are upregulated by environmental stress conditions, possibly those encountered during passage through the gastrointestinal tract. Strains carrying deletions in *inlC2* and *inlD* were found to be unaffected in their ability to invade a number of nonphagocytic cells (Dramsi *et al.*, 1995), hence, the specific functions of these proteins have not yet been defined. Characterization of *lmo0331* and *lmo0610* null mutants has not yet been reported. Considering that a number of σ^B-dependent genes are important in virulence, particularly during gastrointestinal stages of infection (e.g., *opuCA, bsh, inlA*) (Kazmierczak *et al.*, 2003; Sue *et al.*, 2003, 2004; Begley *et al.*, 2005), characterization of null mutations in *inlC2, inlD, lmo0331*, and *lmo0610* in oral or intragastric infection models may provide a

promising approach for identifying the specific functions of these proteins. As species-specificity has been demonstrated for some internalins, e.g., InlA and InlB (Lecuit *et al.*, 1999; Khelef *et al.*, 2006), and as *L. monocytogenes* shows a broad natural host range, characterization in multiple animal species of *L. monocytogenes* strains bearing null mutations in targeted internalin genes may be needed before the specific functions of these internalins can be defined.

lmo2445 represents a newly identified PrfA-dependent internalin gene. Both PrfA-regulated internalins (*lmo2445* and *inlC* [Engelbrecht *et al.*, 1996]) are predicted to encode small secreted internalins lacking surface-anchoring domains (Hamon *et al.*, 2006). Both of these internalin genes are preceded by apparently weak PrfA-binding sites, consistent with a previous report that *inlC* is primarily expressed when PrfA activity is highest, such as when *L. monocytogenes* is inside a host cell (Engelbrecht *et al.*, 1996). It is thus tempting to speculate that both of these secreted internalins may play a role in the later stages of cellular or systemic infection. This hypothesis is consistent with phenotypic characterization, which showed reduced virulence of a Δ*inlC* strain after intravenous infection of mice (Engelbrecht *et al.*, 1996), even though the Δ*inlC* strain showed neither attenuated intracellular replication in J774 or Caco-2 cells (Bergmann *et al.*, 2002) nor attenuated invasion in Caco-2 cells (Engelbrecht *et al.*, 1996). As both of these genes are clearly PrfA-dependent, further characterization of null mutations in both *inlC* and *lmo2445* in different animal and cell culture models appears to be warranted and should allow for further insight into the roles of different internalins in *L. monocytogenes* infection and virulence. Using a number of strains bearing double mutations, Bergmann *et al.* (2002) showed that InlC may have a supportive role in InlA-mediated invasion, suggesting that characterization of dual mutations among internalin genes in a single strain may be needed to further define the function of these internalins.

Sixteen internalin genes, including *inlE, inlF, inlG, inlI,* and *inlJ*, showed no evidence of differential expression by PrfA or σ^B under the conditions used. Consistent with the conclusion that this group of internalins is not regulated by PrfA, Joseph *et al.* (2006) found no evidence of increased expression of alternative internalins in the *L. monocytogenes* intracellular environment. The fact that InlJ has an apparent role in virulence (Sabet *et al.*, 2005) suggests, however, that at least some internalins that do not appear to be regulated by σ^B or PrfA contribute to *L. monocytogenes* virulence.

Conclusions

Listeria monocytogenes is a ubiquitous organism capable of both surviving under diverse environmental conditions and of infecting a wide range of host species and host cell types (e.g., intestinal epithelial cells, endothelial cells [Hamon *et al.*, 2006]). The presence of a large number of *L. monocytogenes* genes encoding cell surface–associated molecules (4.7% of all predicted *L. monocytogenes* genes [Cabanes *et al.*, 2002]) and, in particular, the internalin family, suggests roles for these proteins in interacting with different host and cell types. Internalins represent a diverse family of surface proteins, not only with regard to their structures and function, but also with regard to their transcriptional regulation, as illustrated by the fact that 8 of 24 internalin-like genes in *L. monocytogenes* are regulated by σ^B and/or PrfA, while transcription of the other internalins appears to be regulated by other mechanisms. A comprehensive approach including characterization of null mutant strains (Gaillard *et al.*, 1991; Dramsi *et al.*, 1995, 1997; Bergmann *et al.*, 2002; Sabet *et al.*, 2005), structure determination (Marino *et al.*, 1999; Schubert *et al.*, 2002; Ooi *et al.*, 2006), and transcriptional profiling will be needed to unravel the functions of this group of proteins to ultimately allow a better understanding of their contributions to *L. monocytogenes* survival and transmission in different hosts and environments.

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Address reprint requests to:
Kathryn J. Boor, B.S., M.S., Ph.D.
Department of Food Science
Cornell University
413 Stocking Hall
Ithaca, NY 14853

E-mail: kjb4@cornell.edu