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Genome-Wide Analysis of Lipoprotein Expression in *Escherichia coli* MG1655

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To gain insight into the cell envelope of *Escherichia coli* grown under aerobic and anaerobic conditions, lipoproteins were examined by using functional genomics. The mRNA expression levels of each of these genes under three growth conditions—aerobic, anaerobic, and anaerobic with nitrate—were examined by using both Affymetrix GeneChip *E. coli* antisense genome arrays and real-time PCR (RT-PCR). Many genes showed significant changes in expression level. The RT-PCR results were in very good agreement with the microarray data. The results of this study represent the first insights into the possible roles of unknown lipoprotein genes and broaden our understanding of the composition of the cell envelope under different environmental conditions. Additionally, these data serve as a test set for the refinement of high-throughput bioinformatic and global gene expression methods.

Bacterial lipoproteins comprise a unique set of proteins modified at their amino-terminal cysteines by the addition of *N*-acyl and *S*-diacyl glyceryl groups (30). In *Escherichia coli*, this lipid serves to anchor these proteins to the inner or outer membrane so that they can function at the lipid aqueous interface. These proteins can be identified by the presence of a leader with a common consensus sequence (5). The leader is typically between 15 and 40 amino acid residues in length and has at least one arginine or lysine in the first seven residues. The leader is cleaved by signal peptidase II on the amino terminal side of the cysteine residue, which is then enzymatically modified (30).

The *E. coli* genome has previously been searched for potential lipoproteins. Various algorithms have been used for genome sequence analysis to identify potential lipoproteins, and these lipoproteins have been tabulated in databases on the World Wide Web (http://www.mrc-lmb.cam.ac.uk/genomes/dolop/, http://www.expasy.org/prosite, and http://www.projectcybercell.com); from these databases, we compiled a list of 96 lipoproteins. Fifty-six of these genes (58%) have completely unknown functions, a much higher fraction than that for the *E. coli* genome, in which approximately 25 to 30% of the genes have no known function. Thus, the examination of the expression of the lipoprotein genes under different growth conditions would be a beginning to understanding the function and importance of many of the unknown genes.

Other putative lipoproteins exist in *E. coli* but were not part of the gene expression study. First, the murein transglycosylase MltE (Blattner no. b1163) is not in any current lipoprotein database but has been experimentally shown to be a lipoprotein (17). Second, *yifL* (Blattner no. b3808.1) was originally not annotated in the *E. coli* genome sequencing project, but YifL now appears in the Prosite database (http://www.expasy.org/prosite) as a putative lipoprotein. Also, very small lipoproteins such as the entericidins (EcnA and EcnB) (3) were omitted from this study because they are below the Affymetrix cutoff for open reading frame (ORF) inclusion (150 bp).

In the present study, we used this set of protein genes to begin analyzing the global changes in gene expression during aerobic and anaerobic growth with a view to understanding the changes in the composition of the cell envelope. The expression of lipoprotein mRNAs in *E. coli* MG1655 incubated in glucose defined media (21) either aerobically with shaking in an Erlenmeyer flask or anaerobically in a sealed screw-cap tube, with 40 mM KNO_3 being added to one set of anaerobic cultures as an alternative electron acceptor, was monitored. RNA was then isolated from the cells with a MasterPure RNA purification kit (Epicentre Technologies, Madison, Wis.), and cDNA synthesis and labeling was done as described in the *Affymetrix GeneChip* E. coli *Antisense Genome Array Technical Manual* (1). Affymetrix GeneChip antisense *E. coli* genome arrays were used to analyze the complete *E. coli* transcriptome. Each microarray contained 295,000 probes. Each identified ORF was covered by 15 probe pairs consisting of a perfect match and a 1-nucleotide mismatch pair. If the perfect match probe showed an intensity that was 200 U higher than that of the mismatch probe, the probe pair was considered to be present. An ORF was considered to be present with 95% confidence if neighboring probe pairs within an ORF were present.

Using this cutoff, we were able to group the lipoproteins into four classes, as listed in Table 1. Twenty-one lipoprotein genes were not expressed (not present in the array analysis) under any of the selected conditions. Ten were present under one growth condition, 5 were present under two conditions, and 60 were present under all three conditions. Sixty-four of the lipoprotein genes were expressed at detectable levels during aer-

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TABLE 1. *E. coli* lipoprotein genes and their expression in microarray and RT-PCR analyses[a]

Gene	Blattner no.	Analysis result for growth condition					
		Aerobic		Anaerobic		Anaerobic + nitrate	
		Microarray[b]	RT-PCR	Microarray[b]	RT-PCR	Microarray[b]	RT-PCR
yaeF	b0193	2.85 A	822.74	3.8 A	904.07	5.2 M	709.66
yafL	b0227	13.9 A	149.00	16.15 A	129.12	8.95 A	126.31
yaiW	b0378	39.75 A	1,094.73	39.75 A	1,218.82	46.05 A	1,153.40
ybfP	b0689	4.35 A	112.50	17.9 A	207.54	11.75 A	304.80
ymcA	b0984	2.25 A	370.83	6.05 A	466.07	16.35 M	972.86
ycdR	b1023	0.3 A	38.25	1.2 A	77.86	0.7 A	31.79
ycjN	b1310	12.2 A	23.84	13.1 A	46.44	11.6 A	47.93
wza	b2062	8.65 A	85.28	4.35 A	59.48	4.55 A	75.04
yehR	b2123	1.85 A	250.38	4.65 A	325.93	4.75 A	414.85
yohG	b2138	8.85 A	82.18	19 A	128.07	13.7 A	133.48
yfbK	b2270	15.35 A	19.51	11.6 A	29.78	5.1 A	39.77
ypdI	b2376	0.45 A	108.74	0.35 A	238.47	0.55 A	364.14
yfgH	b2505	5.5 A	256.39	4.1 A	255.68	8.45 A	223.25
yfjS	b2636	3.9 A	165.50	1.8 A	112.20	0.9 A	119.93
yghG	b2971	1.65 A	123.61	1.15 A	82.76	1.1 A	162.09
yghH	b3014	12.6 A	907.25	11.95 A	1,012.54	9.05 A	753.22
acrE	b3265	0.7 A	62.24	0.85 A	58.61	1.35 A	59.62
yiaD	b3552	47.05 M	952.14	24.5 A	603.81	31.35 A	680.38
yiiG	b3896	3.8 A	72.07	3.35 A	107.60	4.8 A	118.86
yjbH	b4029	14.3 A	81.04	14.2 A	147.49	13.35 A	212.67
yjcP	b4080	3.25 A	44.44	4.75 A	81.48	3.85 A	147.26
yaaY	b0024	5.55 A	416.62	10.5 M	998.73	12.9 P	1,417.36
yajI	b0412	20.25 A	300.04	28.65 P	417.83	23.7 A	357.06
ybfN	b0682	15.6 M	357.89	18.55 P	576.64	14.95 M	465.00
yccZ	b0983	25.95 A	341.56	23.15 M	268.29	61.95 P	672.07
ymcC	b0986	15.1 M	192.77	16 M	267.76	47.05 P	2,090.53
csgG	b1037	2.8 A	408.03	18.35 P	1,182.28	12.6 A	581.54
yfiL	b2602	10.35 A	495.61	11.3 A	570.68	11.05 P	368.30
yghJ	b2974	26.6 P	443.18	11.6 A	218.09	9.9 A	187.63
yhiU	b3513	19.4 A	80.34	109.6 P	2,151.94	31.2 A	295.74
yjbF	b4027	27.8 A	58.37	29.9 A	54.12	25 P	45.37
yafT	b0217	15.35 P	344.12	13.7 M	207.16	18.6 P	258.22
ylcB	b0572	83.3 P	2,207.72	11.75 P	128.07	10.25 A	64.35
ybjP	b0865	23.3 P	1,027.23	17.7 A	986.19	58.35 P	1,965.78
yfhM	b2520	22 A	490.08	65.3 P	1,712.68	58.95 P	795.13
slp	b3506	5.6 A	228.51	170.3 P	10,280.10	41.25 P	1,755.04
cutF	b0192	49.65 P	3,464.82	50 P	3,315.12	68.95 P	3,713.77
yaeC	b0197	569.75 P	5,357.99	673.1 P	9,794.30	604.75 P	9,094.88
dniR	b0211	211 P	14,770.97	332.75 P	26,075.12	288.65 P	18,758.77
cyoA	b0432	1,508.05 P	97,254.40	933.95 P	64,118.31	975.1 P	61,809.18
yajG	b0434	467.95 P	18,182.95	322.2 P	11,028.77	286.75 P	8,052.42
ybaY	b0453	43.3 P	10,698.22	52.85 P	19,343.93	137.5 P	8,323.34
acrA	b0463	266 P	21,274.95	262.05 P	20,837.08	256.6 P	10,104.47
ybbC	b0498	12.75 P	1,414.58	23 P	2,338.44	36.15 P	2,893.17
fepG	b0589	68.8 P	5,840.72	57.9 P	4,170.60	70.8 P	7,139.73
rlpA	b0633	172.2 P	8,577.58	173.4 P	6,234.43	168.75 P	3,118.40
rlpB	b0641	336.65 P	16,256.17	344.35 P	18,380.11	280.8 P	13,556.01
ybgE	b0735	80.55 P	4,025.33	625.2 P	37,117.42	622.35 P	26,673.41
pal	b0741	1,088.75 P	85,844.86	858.3 P	72,334.53	737.95 P	48,684.70
ybhC	b0772	184.35 P	4,837.29	152.35 P	4,447.57	133.55 P	3,426.66
yliB	b0830	94.75 P	4,305.95	239 P	16,813.26	195.8 P	8,175.31
yceK	b1050	27 P	2,825.53	32.9 P	4,227.41	35.8 P	5,551.83
yceB	b1063	69.45 P	585.53	101.15 P	1,133.21	106.3 P	481.83
flgH	b1079	431.6 P	22,619.34	274.5 P	12,798.09	115.15 P	5,517.09
ycfL	b1104	90.75 P	8,457.11	121.55 P	11,679.68	160.85 P	12,752.51
ycfM	b1105	113.6 P	3,414.83	185.75 P	6,983.48	225.45 P	9,351.10
lolB	b1209	151.55 P	7,612.79	205.95 P	11,120.93	285.6 P	13,613.65
osmB	b1283	49.1 P	1,493.47	132.65 P	5,617.43	98.75 P	3,191.80
ynbE	b1382	16.25 P	1,639.73	19.4 P	1,998.16	20.3 P	1,368.70
ydcL	b1431	38.25 P	1,839.59	31.85 P	1,342.37	63.35 P	2,357.52
yddW	b1491	13.2 P	327.56	23.5 P	651.77	16.2 P	372.78
ydeK	b1510	34 P	1,152.62	47.85 P	3,366.87	47.3 P	3,367.11

Continued on following page

TABLE 1—*Continued*

Gene	Blattner no.	Analysis result for growth condition					
		Aerobic		Anaerobic		Anaerobic + nitrate	
		Microarray[b]	RT-PCR	Microarray[b]	RT-PCR	Microarray[b]	RT-PCR
slyB	b1641	1,177.4 P	60,695.57	1,052.75 P	61,458.84	1,110.9 P	78,871.69
lpp	b1677	2,142.5 P	652,557.12	1,581.5 P	488,494.78	1,501.85 P	374,766.64
nlpC	b1708	75 P	2,709.71	78.35 P	3,360.72	72.35 P	3,052.40
osmE	b1739	45.55 P	3,368.38	63.3 P	4,086.45	219.75 P	14,538.54
yoaF	b1793	48.6 P	1,424.51	45.65 P	1,424.32	47.3 P	1,586.21
yeaY	b1806	107.7 P	4,334.04	86 P	2,973.40	130.3 P	2,282.15
yebF	b1847	136.6 P	3,104.08	132.2 P	3,019.95	197.15 P	5,182.22
yecR	b1904	126.4 P	14,835.41	25.95 P	1,935.43	17.15 P	940.23
yedD	b1928	172.1 P	12,536.98	171.3 P	11,952.42	160.55 P	19,869.35
spr	b2175	587.4 P	46,027.52	233.45 P	17,688.27	220.05 P	14,997.48
rtn	b2176	56.55 P	952.64	50.45 P	874.74	48.65 P	664.40
yojL	b2214	46.1 P	872.69	59.3 P	1,142.29	61.25 P	877.69
vacJ	b2346	220.5 P	3,966.98	167.4 P	3,049.50	159.7 P	2,716.95
yfeY	b2432	96.85 P	6,019.97	114.6 P	6,788.17	245.15 P	12,690.81
nlpB	b2477	354 P	11,015.25	426.5 P	16,943.41	392.3 P	10,879.40
yfgL	b2512	313.4 P	21,018.48	293.95 P	23,681.22	328.35 P	27,646.47
yfiH	b2593	85.1 P	4,998.32	95.3 P	6,011.15	101.95 P	5,639.94
yfiO	b2595	332.25 P	15,555.04	343.8 P	17,027.70	376.75 P	16,355.48
yfiB	b2605	64.05 P	3,947.83	77.95 P	3,417.15	73.05 P	4,036.95
mltB	b2701	41.95 P	560.42	65.95 P	600.54	71.65 P	656.93
nlpD	b2742	1,018.55 P	54,658.96	1,397.25 P	93,860.15	1,480.25 P	57,912.34
ygdI	b2809	10.9 P	593.84	15.5 P	1,113.41	52.8 P	2,160.82
mltA	b2813	64 P	1,918.08	75.35 P	2,154.47	91.3 P	1,910.28
ygdR	b2833	49.05 P	1,914.44	50.15 P	1,576.61	60.65 P	2,155.33
ygeR	b2865	42.3 P	501.07	50.1 P	573.73	48.2 P	348.11
mltC	b2963	126.6 P	3,392.37	101.15 P	2,259.74	99.35 P	1,746.64
yraP	b3150	119.05 P	5,530.13	108.25 P	6,152.70	174.3 P	8,350.46
nlpI	b3163	1,415.7 P	197,056.23	1,460.15 P	299,438.99	1,470.4 P	281,225.58
yhdV	b3267	28 P	582.55	28.45 P	815.84	21 P	260.17
yhfL	b3369	11.8 P	338.64	11.45 P	478.89	12.05 P	406.85
nlpA	b3661	62.9 P	2,889.24	78 P	4,949.62	94.3 P	5,323.51
blc	b4149	31.25 P	1,599.72	22.8 P	1,090.51	56.25 P	1,189.99
yjfO	b4189	26.9 P	968.41	19.9 P	963.99	25.45 P	966.50
fecD	b4288	15.85 P	593.54	11.85 P	392.53	12.3 P	336.81
rcsf	b0196	220.45 P		232.75 P		263.2 P	
borD	b0557	98.2 P		117.5 P		62.85 P	
ybjR	b0867	48.35 P		49.85 P		71.2 P	
ycaL	b0909	15.1 P		23.95 P		23.2 P	
mltE	b1193	106.5 P		104.2 P		89.6 P	
hslJ	b1379	35.1 P		75.15 P		77 P	
ynfC	b1585	25 P		17 P		19.5 P	
smpA	b2617	199.45 P		196.35 P		257.5 P	
yggG	b2936	47.3 P		32.8 P		34.25 P	
yidQ	b3688	86.85 P		164.35 P		227 P	
yidX	b3696	8.6 P		6.4 P		7.85 P	
yjeI	b4144	228.5 P		182.9 P		216.4 P	

[a] Genes are divided into four groups based on the microarray expression profile described in the text. RT-PCR measurements were made as described in the text, and data are expressed as the gene copy number in the presence of reverse transcriptase minus the gene copy number in the absence of reverse transcriptase and are normalized to 10 μg of mRNA. Each value is the average for two experiments.

[b] mRNA status as determined by analysis with Affynaetrix Suite 5.0 software: P, present with 95% confidence; A, absent; M, marginal (present with 94% confidence).

obic growth, the standard experimental growth condition for *E. coli*. Not surprisingly, *lpp*, the gene for the major structural outer membrane murein lipoprotein (25), has the highest expression level of all the genes. Other well-known lipoprotein genes highly expressed under aerobic growth conditions include *pal*, the gene for peptigoglycan-associated lipoprotein (19), and *cyoA*, which encodes a subunit of the cytochrome O terminal oxidase, the major terminal oxidase of the aerobic respiratory chain (7).

We then used real-time PCR to help better quantify the expression levels for the lipoprotein genes. First, reverse transcription (RT) was carried out with the same total RNA samples used for the microarray analysis and random hexamer primer (Invitrogen, Burlington, Ontario, Canada). RT was performed with SuperScript II (Invitrogen) for reactions with RT (+RT reactions). Control reactions were also performed under the same conditions except that SuperScript II was omitted (−RT reactions). Both types of reactions were used in real-time PCRs.

Primers for real-time PCR were designed with Primer Express 2.0 software from Applied Biosystems (ABI) (Foster City, Calif.). Forward and reverse primer pairs were designed

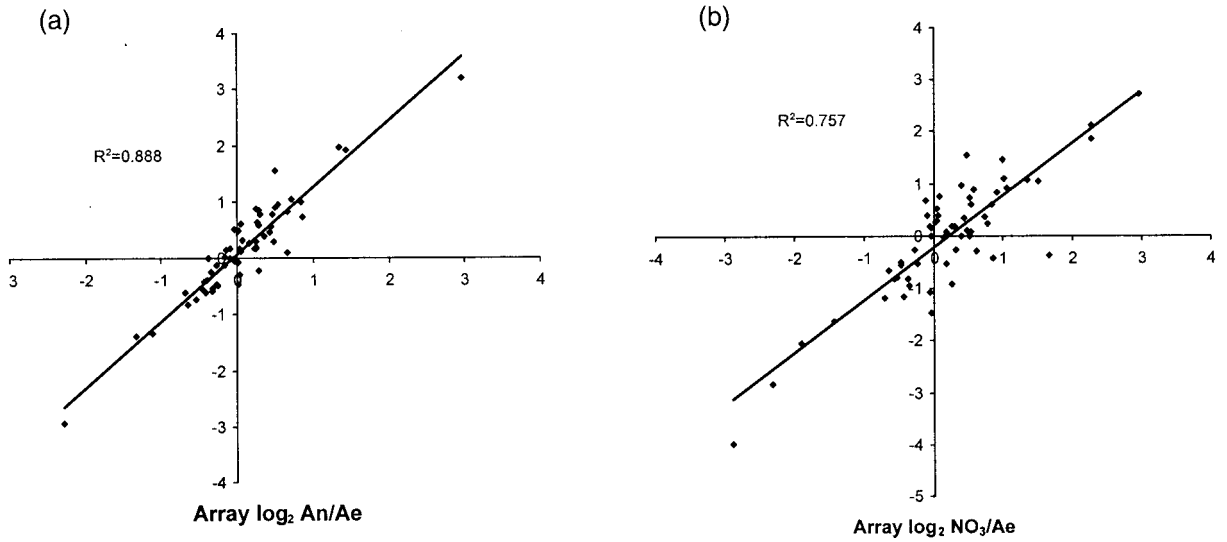

FIG. 1. Scatter plot comparative analysis of microarray and RT-PCR data. The signal intensities of 61 genes which were designated "present" under all three growth conditions in the microarray data set were compared to the signal intensities generated by RT-PCR. Each datum point represents the $\log_2$ ratio of the signal intensity determined for one growth condition to the signal intensity determined for another growth condition, as determined by both RT-PCR and microarray analyses. (a) Ratios for anaerobically (An) versus aerobically (Ae) grown cells; (b) ratios for anaerobic-plus-nitrate (NO_3) versus aerobic cells.

for the 5′ and 3′ regions of each gene and purchased from Sigma Genosys (Oakville, Ontario, Canada). Real-time PCRs were carried out for each primer set with both the +RT and −RT reactions for each growth condition. The reaction buffer contained, in part, 1× ROX glycine conjugate of 5-carboxy-X-rhodamine, with succinimidyl ester as the inert-passive reference dye, and SYBR Green I. The reaction mixtures were aliquoted into 384-well ABI reaction plates. The plates were then placed in an ABI Prism 7900HT RT-PCR machine under the following conditions: stage 1 consisted of 95°C for 45 s; stage 2 consisted of 40 cycles of 95°C for 15 s, followed by 60°C for 1 min; stage 3 consisted of 95°C for 15 s; stage 4 consisted of 60°C for 15 s; and for stage 5, the temperature was ramped to 95°C for 5 s. The RT-PCR data were analyzed with SDS 2.0 software (ABI). Each +RT-versus-−RT reaction set was compared against a standard curve generated for each primer set by using *E. coli* linear DNA as a standard. A cycle threshold value was chosen that gave a linear regression value greater than 0.996 for each primer set standard curve. The calculated quantity values for each +RT or −RT reaction were standardized within each individual primer set-generated standard curve.

The RT-PCR data correlate well with the microarray data in that highly expressed genes found in the microarray study also give high RT-PCR signals. However, the RT-PCR results are much more accurate and sensitive and give a wider dynamic range of numbers. Signal intensity ratios for anaerobic-versus-aerobic and anaerobic-plus-nitrate-versus-aerobic data sets were calculated for both microarray ("present" values only) and RT-PCR data and are compared in a scatter plot in Fig. 1. The anaerobic/aerobic ratios had very good correlation between RT-PCR and microarray data, with an R^2 value of 0.888; the nitrate/aerobic ratios had a slightly lower correlation (R^2 = 0.757).

A further examination of gene expression patterns based on the RT-PCR data was then undertaken in order to gain some insight into possible functions of unknown lipoproteins. Growth under anaerobic conditions results in significant changes in the expression of many of the lipoprotein genes relative to aerobic expression. The expression of key structural protein genes such as *lpp* and *pal* remained fairly constant under these conditions, which was expected given their "housekeeping" role. However, transcripts of 14 genes are induced twofold or more under anaerobic growth. The gene with the strongest anaerobic induction is *slp* (Table 1). This gene is known to be induced under conditions of starvation or in the stationary phase (2), so perhaps it is not surprising that it is also induced during slow anaerobic growth. Other genes with strong anaerobic induction include *ybgE*, which appears to be cotranscribed with the *cydAB* cytochrome D terminal oxidase (also strongly induced anaerobically [data not shown]), and *osmB*, a lipoprotein gene which is also induced by high osmotic strength and in the stationary phase (15, 16). Only five genes had twofold or greater reductions of signal intensity under anaerobic conditions relative to that under aerobic conditions. These genes included *cusC/ibeB*, which is induced by high concentrations of copper ions (20) and appears to be important for virulence and invasion across the blood-brain barrier for other *E. coli* strains (12, 13), and *spr*, which encodes a putative penicillin binding protein (11).

The addition of nitrate to an anaerobic culture as an alternative electron acceptor also influences the expression of several of the lipoprotein genes. It is known that the addition of nitrate to anaerobic cultures regulates the expression of many genes, especially those involved in alternative electron transport pathways (26). When grown anaerobically with nitrate, the RT-PCR signals for 13 genes decreased twofold or more and those for 4 genes increased twofold or more relative to those under anaerobic conditions without nitrate. Anaerobically induced genes such as *slp* and *yhiU* are repressed with the addi-

tion of nitrate. Two of the four lipoproteins induced by the addition of nitrate to an anaerobic culture, albeit with low overall signals, are *ymcA* and *ymcC*, which form part of a putative *ymcCBA* operon. Another gene induced by nitrate is *osmE*, a putative lipoprotein gene which is induced by high osmotic strength (10).

Microarrays have fast become a commonly used tool to examine global expression profiles for many bacterial species (see reference 6 for a recent review). Many of these microarray studies have gone one step further by selection of a subset of genes to determine expression by RT-PCR data, which are then compared to the microarray data (4, 8, 9, 18, 22–24, 27–29). However, in these cases, the genes studied by RT-PCR are chosen as a subset of genes of interest that were originally identified by the microarray assay. The approach presented here is different; the gene set to be studied by RT-PCR was not chosen on the basis of the microarray results; instead, it was chosen based on known or predicted functions of the gene products. Even with this unbiased approach to selecting genes for RT-PCR analysis, the correlation between the RT-PCR data and the microarray data is very good. The more accurate and quantitative RT-PCR data were not used just for comparative purposes, however. These data were then used to identify potentially significant unknown lipoprotein genes with either high gene expression levels or significant changes in gene expression depending on growth conditions. This study has produced the first real data reported for these unknown genes and may lead to more effective investigation of these genes in the future. With the usefulness of this approach assured, it is now time to further study the other unknown lipoprotein genes showing either strong or varied expression levels by other means.

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ADDENDUM

As this paper was under review, other potential lipoproteins in *E. coli* came to our attention, especially those predicted in a recent related paper (14). These genes—*rcsF* (Blattner no. b0196), *borD* (b0557), *ybjR* (b0867), *ycaL* (b0909), *hslJ* (b1379), *ynfC* (b1585), *smpA* (b2617), *yggG* (b2936), *yidQ* (b3688), *yidX* (b3696), and *yjeI* (b4144)—are included in the microarray results, but RT-PCR data were not generated.

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