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Pasteurella multocida Gene Expression in Response to Iron Limitation

MICHAEL L. PAUSTIAN, BARBARA J. MAY, AND VIVEK KAPUR*

Department of Veterinary Pathobiology and Biomedical Genomics Center, University of Minnesota, St. Paul, Minnesota 55108

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Pasteurella multocida **is the causative agent of a wide range of diseases in avian and mammalian hosts. Gene expression in response to low iron conditions was analyzed in *P. multocida* using whole-genome microarrays. The analysis shows that the expression of genes involved in energy metabolism and electron transport generally decreased 2.1- to 6-fold while that of genes used for iron binding and transport increased 2.1- to 7.7-fold in *P. multocida* during the first 2 h of growth under iron-limiting conditions compared with controls. Notably, 27% of the genes with significantly altered expression had no known function, illustrating the limitations of using publicly available databases to identify genes involved in microbial metabolism and pathogenesis. Taken together, the results of our investigations demonstrate the utility of whole-genome microarray analyses for the identification of genes with altered expression profiles during varying growth conditions and provide a framework for the detailed analysis of the molecular mechanisms of iron acquisition and metabolism in *P. multocida* and other gram-negative bacteria.**

Pasteurella multocida is a gram-negative, nonmotile, rod-shaped, facultative anaerobe that has been isolated from a wide range of mammals and birds throughout the world. This organism is the etiologic agent of a variety of economically significant diseases, including fowl cholera in poultry, hemorrhagic septicemia in cattle and buffalo, atrophic rhinitis in swine, and snuffles in rabbits (9, 12). The global distribution, severity of disease caused, and the wide variety of livestock affected by *P. multocida* account for considerable economic losses due to this pathogen worldwide (12).

Iron is an essential nutrient for most organisms due to its important role in metabolic electron transport chains. Due to the presence of specialized protein carriers such as transferrin and lactoferrin in body fluids, the concentration of free iron normally present in mammalian and avian hosts is not enough to support the in vivo growth of bacteria (1, 2). Successful pathogens must therefore possess an effective response to the limited iron conditions encountered upon entry into a host. Previous studies illustrate the fact that the identities of genes and pathways involved in the acquisition, transport, and utilization of iron in *P. multocida* are poorly understood (5, 6, 10, 11, 13, 16). Here we have utilized whole-genome microarray analysis to identify genes with altered expression patterns when *P. multocida* is grown under iron-limiting conditions.

Bacterial growth and RNA isolation. *P. multocida* PM70 was grown to log phase in a flask of brain-heart infusion (BHI) medium (Becton Dickinson) at 37°C. The culture was split into two 180-ml volumes, briefly centrifuged at 4°C, washed with 1× phosphate-buffered saline (pH 7.0), and centrifuged again. One pellet was resuspended in 180 ml of BHI medium, and the other was resuspended in 180 ml of BHI medium containing the iron chelator 2,2′-dipyridyl (200 μM) (Sigma, St. Louis, Mo.). The resuspended cultures were incubated on a rotary shaker at 37°C, and 30-ml volumes were removed 15, 30, 60, and 120 min after resuspension. These samples were briefly centrifuged at 4°C, and the pellets were flash frozen in dry ice and ethanol. Total RNA extractions were performed with RNeasy Maxi columns (Qiagen, Chatsworth, Calif.), with DNase digestions done on the column by adding 82 Kunitz units of enzyme (Qiagen) and incubating at room temperature for 15 min.

Microarray analysis. Gene expression analysis with DNA microarrays was performed as described elsewhere (http://www.cbc.umn.edu/ResearchProjects/AGAC/Pm/Pmarraydata.html) (8a). In brief, a library of targets representing all 2,014 open reading frames (ORFs) from *P. multocida* PM70 (AE004439) was constructed with primers designed to amplify fragments of ≤500 bp from each ORF from genomic DNA. Two successive rounds of PCR were performed to minimize genomic DNA contamination in the products of amplification, and the final 100-μl reactions were checked for quality on agarose gels and purified with MultiScreen PCR plates (Millipore, Bedford, Mass.). The 1,936 (96%) ORF segments that were successfully amplified were printed using a Total Array System robot (BioRobotics, Boston, Mass.). RNA from *P. multocida* grown in BHI medium alone or BHI medium containing 2,2′-dipyridyl were labeled with Cy3 and Cy5, respectively, and competitively hybridized with the printed microarrays. Images of the hybridized arrays were obtained with a Scanarray 5000 microarray scanner (GSI Lumonics, Watertown, Mass.). Two independent hybridizations using independent RNA extractions were performed for each time point. Fluorescent intensities for individual spots were normalized based on the total intensity of fluorescence in the Cy3 and Cy5 channels. Hierarchical clustering and analysis were performed using the publicly available programs Cluster and Treeview (M. Eisen; http://www.microarrays.org/software).

* Corresponding author. Mailing address: 1971 Commonwealth Ave., St. Paul, MN 55108. Phone: (612) 625-7712. Fax: (612) 625-5203. E-mail: vkapur@umn.edu.

TABLE 1. *P. multocida* genes with alterations in expression level of at least twofold in response to low iron conditions

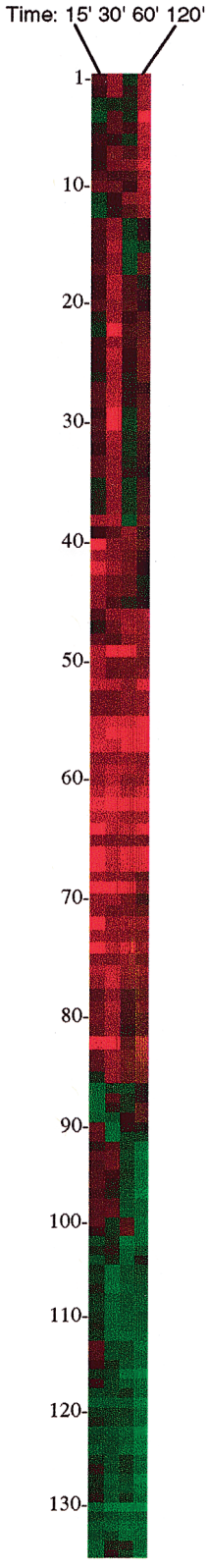
ORF	Gene	Description	Fold change[a] in expression level
576	*hemR*	Heme-hemopexin utilization protein C	10.6
639	*rpL25*	Ribosomal protein L25	8.2
288	*lldD*	L-Lactate dehydrogenase	7.8
42		Hypothetical HI0854 protein	7.7
336		Putative TonB-dependent receptor	7.7
399	*yfeB*	Iron(III) dicitrate transport ATP-binding protein	7.2
299		Hypothetical HI0854 protein	6.2
300		Putative TonB-dependent receptor	6.0
456	*ponB*	Penicillin-binding protein 1B	5.6
779	*hexC*	HexC, capsule biosynthetic locus	5.4
1078		Hemin binding receptor	5.2
51	*fbpA*	Iron binding protein FbpA precursor	5.0
400	*yfeA*	Mn transport protein, ABC transporter	5.0
1459	*pgtC*	Phosphoglycerate transport regulatory protein	4.9
803		Hypothetical HI1369 protein	4.8
1186	*exbB*	Biopolymer transport protein homolog	4.7
121	*hpt*	Hypoxanthine phosphoribosyltransferase	4.6
131	*fecB*	Iron(III) dicitrate-binding periplasmic protein	4.3
122	*gmhA*	Phosphoheptose isomerase	4.0
398	*yfeC*	Iron (chelated) ABC transporter	4.0
129	*fecD*	Iron(III) dicitrate transport system permease	3.6
1079		FecCD family membrane transport	3.6
668	*fnr*	Fumarate (and nitrate) reduction regulatory protein	3.6
1802		Hypothetical Hl0847 protein	3.4
130	*fecC*	Iron(III) dicitrate transport system permease	3.4
1460		Phosphoglycerate transport system activator protein	3.3
1205	*hisH*	Amidotransferase	3.3
397	*yfeD*	Iron (chelated) transporter, permease protein	3.1
357	*menE*	*o*-Succinylbenzoate–CoA ligase	3.1
1748	*hslV*	Putative heat shock protein	3.0
212		Hypothetical HI1601 protein	2.9
1842		Unknown	2.9
424		Hypothetical Hl1736 protein	2.9
583	*trpG*	Anthranilate synthase component II	2.9
1639	*tal*	Transaldolase	2.9
1481		Unknown	2.8
1456		Putative ATP-binding transport protein	2.8
1095		Unknown	2.8
1457		Putative periplasmic iron-binding protein	2.7
452		Hypothetical *Yersinia pestis* protein	2.6
1816	*recX*	Regulatory protein, RecX homolog	2.6
1474	*cydD*	Transport ATP-binding protein	2.6
741		Hemoglobin receptor precursor	2.5
1188	*tonB*	TonB protein	2.5
1697	*folB*	Dihydroneopterin aldolase	2.5
804	*pqqL*	Zinc protease	2.5
734	*htrA*	Heat shock protein, periplasmic serine protease	2.5
883		Hypothetical Hl1293/94 protein	2.5
1529	*hpaG*	2-Hydroxyhepta-2,4-diene-1,7,-dioate	2.5
1627	*ilvM*	Acetolactate synthase small subunit	2.5
719	*nrdB*	Ribonucleoside diphosphate reductase, beta chain	2.5
720		Hypothetical Hl1309	2.5
298		Hypothetical *Shigella dysenteriae* protein	2.4
1722		Hypothetical Hl0857 protein	2.3
839	*aroA*	3-Phosphoshikimate 1-carboxyvinyltransferase	2.3
213		Hypothetical Hl1602 protein	2.3
50	*fbpB*	Putative transmembrane permease	2.3
1348	*slyX*	SlyX protein homolog	2.3
611	*sbcB*	Exonuclease I	2.3
1187	*exbD*	Biopolymer transport protein	2.3
451		Hypothetical *Y. pestis* protein	2.3
90		Unknown	2.2
1730	*glmS*	Glucosamine–fructose-6-phosphate aminotransferase	2.2
875	*nagB*	Glucosamine-6-phosphate isomerase	2.2
1080		Hypothetical *S. dysenteriae* protein	2.2
1226	*comD*	Putative competence D protein	2.2
656	*tehB*	Tellurite resistance protein	2.2
353	*fldA*	Flavodoxin	2.2

Continued on following page

TABLE 1—*Continued*

ORF	Gene	Description	Fold change[a] in expression level
1483		Hypothetical Hl0668 protein	2.2
49	*fbpC*	ATPase, ABC-type transport protein	2.2
1171		Hypothetical *Escherichia coli* protein	2.2
1142	*rpL31*	Ribosomal protein L31	2.2
1163	*mpA*	RNase P	2.1
805		Unknown	2.1
723	*ttrB*	Tetrathionate reductase subunit B	2.1
713		Hypothetical HI1333 protein	2.1
1727		ATP-binding protein, ABC transporter	2.1
1604		Hypothetical *E. coli* protein	2.1
91		Hypothetical HI0883 protein	2.0
317		Hypothetical Hl0379 protein	2.0
1201		Unknown	2.0
1473		Putative ABC transporter, ATP-binding protein	2.0
1993	*skp*	Skp, lipid A biosynthesis	2.0
41		Hypothetical ABC transporter, ATP-binding protein I	2.0
1761		ORF-3, hypothetical *Streptococcus pneumoniae* protein	2.0
1792		Pentahemic *c*-type cytochrome	−2.0
1026	*sprT*	SprT homolog	−2.0
1434	*dcuB*	Anaerobic dicarboxylate transport protein homolog	−2.0
587		Hypothetical *E. coli* protein	−2.0
1331		Putative NADH:ubiquinone oxidoreductase	−2.0
823	*fumC*	Fumarate hydratase class II	−2.1
1379		Ribose ABC transporter, ATP-binding protein	−2.1
832	*ptnD*	Phosphotransferase system enzyme II	−2.1
667	*rsgA*	Ferritin-like protein 2	−2.1
1696		Hypothetical HI0266 protein	−2.2
1211		Hypothetical HI1048 protein	−2.2
1592	*napF*	Ferredoxin-type protein	−2.3
1491	*atpH*	ATP synthase, delta chain	−2.3
1854		Putative iron sulfur protein	−2.3
1374		Unknown	−2.3
409	*fdxG*	Formate dehydrogenase, alpha major subunit	−2.3
1286	*uspA*	Universal stress protein, phosphotransferase	−2.4
897	*ptsl*	Phosphoenolpyruvate protein, phosphotransferase	−2.4
834		Phosphotransferase system enzyme II	−2.4
1299		Hypothetical HI0585 protein	−2.4
81		Hypothetical HI0902 protein	−2.4
898	*ptsH*	Phosphocarrier protein Hpr	−2.4
408	*fdxG*	Formate dehydrogenase, alpha major subunit	−2.5
75	*pflB*	Formate *c*-acetyltransferase	−2.5
1480		Hypothetical *E. coli* protein	−2.5
942		Hypothetical Hl0081 protein	−2.5
1372		Putative sugar kinase	−2.6
641	*bioD1*	Dethiobiotin synthase	−2.6
74		Putative formate transporter	−2.6
1860	*pgk*	Phosphoglycerate kinase	−2.6
406	*fdxl*	Formate dehydrogenase, gamma subunit	−2.6
1754	*dmsA*	Anaerobic dimethyl sulfoxide reductase, chain A	−2.6
1285	*mazG*	MazG protein	−2.8
1871	*eno*	Phosphopyruvate hydratase (enolase)	−2.8
407	*fdxH*	Formate dehydrogenase, beta subunit	−2.8
666	*rsgA*	Ferritin-like protein 1	−2.9
431	*phoR*	Phosphate regulon sensor protein	−2.9
219	*arcA*	Aerobic respiration control protein	−3.0
1852	*lctP*	L-Lactate permease	−3.2
924	*gapdH*	Glyceraldehyde-3-phosphate dehydrogenase	−3.3
1212	*merT*	Putative mercuric ion transport protein	−3.3
1378		Ribose ABC transporter, permease protein	−3.5
198	*frdD*	Fumarate reductase, 13-kDa hydrophobic protein	−3.7
1377	*rbsB*	D-Ribose binding periplasmic protein precursor	−3.8
199	*frdC*	Fumarate reductase, 15-kDa hydrophobic protein	−3.8
64		Hypothetical Hl0017 protein	−3.8
1453	*adh2*	Alcohol dehydrogenase 2	−4.4
201	*frdA*	Fumarate reductase, flavoprotein subunit	−5.5
200	*frdB*	Fumarate reductase, iron sulfur protein	−6.0
331	*ompW*	Outer membrane protein OmpW precursor	−6.5

[a] Fold change is the maximum value observed over four time points. Negative values indicate decreases in the expression level.



Profile Number	ORF	Profile Number	ORF	Profile Number	ORF
1	1483	46	741	91	431
2	720	47	452	92	1372
3	839	48	317	93	1378
4	639	49	336	94	1374
5	357	50	1816	95	1285
6	1842	51	719	96	1754
7	1529	52	131	97	1377
8	668	53	875	98	1379
9	213	54	451	99	587
10	212	55	399	100	1860
11	734	56	42	101	1286
12	91	57	400	102	1212
13	1761	58	298	103	1211
14	1201	59	130	104	1792
15	1481	60	397	105	407
16	1171	61	398	106	406
17	805	62	288	107	1852
18	1748	63	1205	108	1331
19	1456	64	299	109	408
20	713	65	1473	110	1854
21	1627	66	1078	111	409
22	456	67	51	112	641
23	1722	68	1188	113	1871
24	1802	69	576	114	823
25	583	70	1187	115	1480
26	1697	71	1080	116	331
27	1639	72	1186	117	1299
28	1460	73	1474	118	832
29	779	74	300	119	64
30	1459	75	353	120	75
31	1457	76	1079	121	898
32	883	77	129	122	200
33	723	78	1727	123	198
34	90	79	424	124	199
35	1226	80	1730	125	834
36	1604	81	611	126	1491
37	1142	82	803	127	1453
38	1095	83	1348	128	924
39	1993	84	1163	129	897
40	121	85	656	130	201
41	804	86	1696	131	1434
42	122	87	1592	132	219
43	50	88	81	133	74
44	49	89	1026	134	667
45	41	90	942	135	666

FIG. 1. Hierarchical clustering of 135 *P. multocida* ORFs that had significantly altered expression levels under low iron conditions. Clustering and visualization were performed using the software programs Cluster and Treeview. Red and green colors represent fold increase and decrease, respectively, in gene expression in response to low iron conditions. The gene expression profiles for the 15-, 30-, 60-, and 120-min time points are shown in the panel on the left, and corresponding ORF numbers in the *P. multocida* genome are also shown.

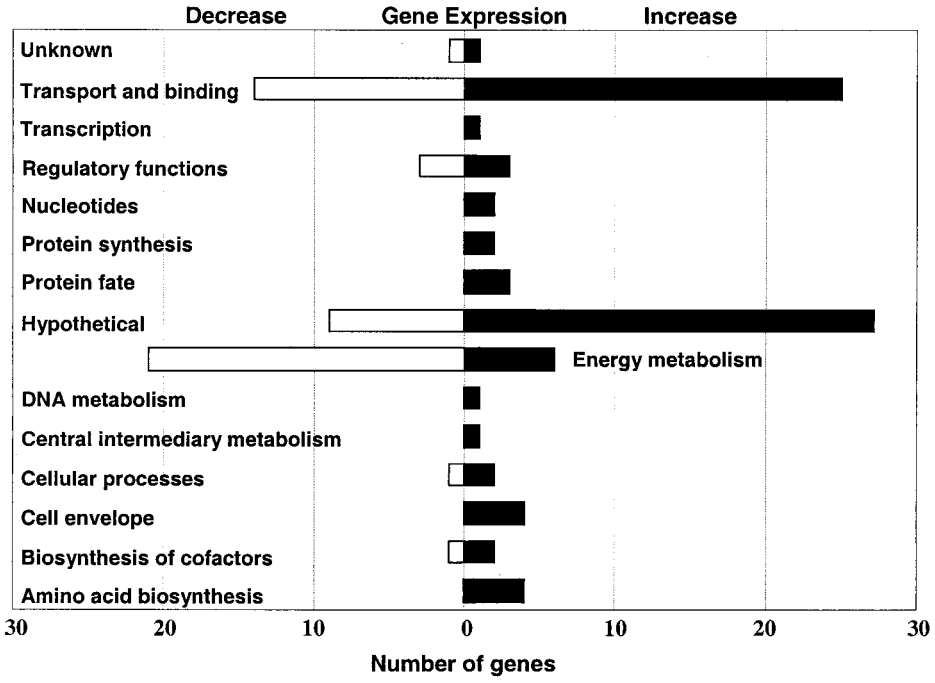


FIG. 2. Functional classification of *P. multocida* genes that had altered expression levels, of twofold or more, in response to low iron conditions. Open and closed bars represent the numbers of genes that decreased and increased in expression, respectively.

***P. multocida* genes with altered expression profiles.** A complete set of all results is presented on our website at http://www.cbc.umn.edu/ResearchProjects/AGAC/Pm/Pmarraydata.html. Of the 1,936 ORFs represented on the array, 135 had changes in expression of at least twofold over the course of the experiment (Table 1 and Fig. 1). The remaining ORFs either were not well measured or did not have detectably altered expression levels. A twofold change in the level of expression was used as an indication of significance based on the reproducibility of results obtained in our lab. These genes can be functionally classified based on homology with previously described proteins in public databases (Fig. 2). The classification strategy we used was based on the system developed for the *Haemophilus influenzae* genome (4). Several genes involved in energy metabolism had altered expression levels. For example, three genes encoding glycolysis enzymes (*gapdh, pgk*, and *eno*) had, on average, 2.8-fold decreases in expression in response to low iron conditions, while lactate dehydrogenase expression increased nearly 8-fold. The gene encoding Fnr, a transcriptional activator of genes involved in anaerobic metabolism, was expressed at 3.6-fold higher levels. Interestingly, some genes that are normally transcriptionally activated by Fnr, such as fumarate reductase and formate dehydrogenase (*frdABCD* and *fdxGHI*), had an over fourfold decrease in average expression. Additionally, the hypothetical protein encoded by ORF 0064 decreased 3.8-fold and is homologous to YfiD, a pyruvate formate lyase also regulated by Fnr. These discrepancies in Fnr regulation may be due to the disruption of FeS cluster cofactors that Fnr has been proposed to utilize for oxygen sensing (8). The expression of *arcA*, a transcriptional repressor of aerobic metabolism genes, decreased nearly threefold. In contrast, four genes involved in amino acid biosynthesis (*aroA, trpG, hisH*, and *ilvM*) had an average increase in expression of 2.6-fold. Together, these data show that in general the expression levels of genes involved in energy metabolism decreased 2.1- to 6-fold while those of genes involved in DNA and central intermediary metabolism, as well as those involved in amino acid biosynthesis, generally increased (Fig. 2).

As expected, many genes encoding proteins involved in iron transport increased their expression levels from 2.1- to 7.5-fold in response to low iron conditions (Table 1). These genes included *yfeABCD, fbpABC, fecBCD, tonB*, and *exbBD*. The Yfe, Fbp, and Fec systems are involved in the transport of iron into the cytoplasm, while TonB and ExbBD provide the energy for this to occur. Interestingly, five genes homologous to ABC transport proteins also had an average increase in expression of 2.3-fold. These may represent additional, uncharacterized bacterial transport systems that appear to be regulated by cellular iron content. Additionally, 10 genes involved in the transport of carbohydrates had significantly decreased expression (Table 1). This observation may be related to the decrease in the expression of genes involved in energy metabolism.

Several stress response genes had altered expression levels under low iron conditions. One of these, *recX*, increased its expression 2.6-fold and has been shown to play a role in the SOS response by binding and inactivating RecA, which normally triggers DNA repair and mutagenesis during an SOS response (17). RecA itself did not have altered expression, but transcription of the universal stress protein A gene (*uspA*) decreased 2.3-fold. Recent work has shown that *uspA* may be positively regulated in part by *recA* (3), and therefore it is possible that the relative excess of RecX may be contributing to a decrease in RecA activity. The expression of the heat shock proteins HslV and HtrA also increased an average of

TABLE 2. Hypothetical genes in close proximity to known genes that also had altered expression levels[a]

ORF cluster	Function of known gene(s)
1211, 1212	Mercuric ion transport
212, **213**	Hypothetical
298, **299**, 300	TonB-dependent receptor
803, 804, **805**	Zinc protease
1078, 1079, **1080**	Hemin binding receptor
1456, 1457	Putative ABC transporter

[a] Hypothetical genes are shown in bold.

2.8-fold in response to low iron. HtrA has been shown to respond to periplasmic stress (15), while HslV may be involved in the regulation of cell division protein SulA (14). Two ribosomal proteins, rpL25 and rpL31, had increases in expression of 8- and 2.1-fold, respectively, under low iron conditions. These changes may represent global responses to iron deprivation. Four genes involved in cell surface biosynthesis (*gmhA, skp, hexC*, and *ponB*) were also expressed at levels that were an average of fourfold higher than controls. The increase in expression of both stress-related and cell surface synthesis genes indicates that the iron chelator may have had a detrimental effect on the bacterial membrane or that the low iron conditions resulted in modifications and alterations in the bacterial cell surface.

Among the most noteworthy findings was the observation that 27% of the genes with significantly altered expression levels encode hypothetical proteins or have homology to hypothetical proteins from other bacteria (Fig. 2). These results reveal the limitations of our current understanding of genes involved in major processes in bacterial growth and metabolism. Interestingly, many of these hypothetical genes are physically located next to genes with homology to proteins with known functions that also had altered expression profiles (Table 2). This suggests the possibility that some of these hypothetical proteins are coregulated with these previously characterized genes and may possess similar or complementary functions. For example, the hypothetical protein encoded by ORF 0803 appears to have a TonB-dependent outer membrane receptor motif at the C-terminal end. Expression of ORF 0803 increased 4.8-fold, while ORF 0804 (a putative zinc protease) expression in *P. multocida* increased 2.5-fold. It is possible that these proteins may function together to bind and degrade iron-containing proteins such as transferrin, a hypothesis that remains to be directly tested (7).

Utility of the microarray-based approach for profiling of gene expression in bacteria. A major advantage of the microarray approach is that it enables simultaneous profiling of the transcriptional activity of the entire bacterial genome in a time- and cost-efficient manner. This is especially important in ascribing possible functions to the many hypothetical genes discovered through the whole genome sequencing approach. Furthermore, knowledge of the transcriptional profile of the entire genome enables a holistic approach to the understanding of the metabolic state of the organism under various conditions. Apart from being relatively inexpensive to set up and perform, the use of two-color hybridizations permits comparative analyses of bacterial gene expression by obviating many of the sources of variation inherent in other methods, including single color- or radiolabel-based hybridization methods and many of the PCR-based approaches.

However, there are numerous limitations that must be considered when interpreting results of microarray-based expression analyses. For example, the analyses are limited in that they only index changes in the transcription of a gene and do not account for posttranscriptional regulation that may influence gene and protein expression. Furthermore, short-lived and unstable transcripts are often not well measured since microarrays are essentially a "snapshot" of the transcriptional activity at a fixed time point. The sensitivity of microarrays to detect small changes in gene expression is also currently unknown. Therefore, the results of microarray analyses need to be confirmed with more sensitive techniques such as quantitative PCR-based approaches both for validation purposes and to minimize the occurrence of "false positives." Overall, it is important to note that these limitations do not detract from the overall utility of the microarray-based approach for global gene expression profiling in bacteria. Therefore, while microarrays do not provide the definitive answer to all questions of gene expression and regulation in bacterial pathogens, they serve as an excellent starting point for screening large numbers of genes to determine patterns of differential gene expression and to compare transcript profiles of bacterial cells of differing phenotypes or those that are subject to different environmental stimuli.

In summary, the results of our investigations show that microarray-based analysis of gene expression provides an effective tool for the identification of gene targets that are involved in major metabolic processes in bacterial pathogens as well as in the initial stages of infection and iron acquisition from hosts.

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