JOURNAL OF BACTERIOLOGY, Aug. 2006, p. 5640–5645 Vol. 188, No. 15
0021-9193/06/$08.00+0 doi:10.1128/JB.00417-06

Transcriptional Profile of *Haemophilus influenzae*: Effects of Iron and Heme

Paul W. Whitby,[1] Timothy M. VanWagoner,[1] Thomas W. Seale,[1] Daniel J. Morton,[1] and Terrence L. Stull[1,2]*

Departments of Pediatrics[1] and Microbiology/Immunology,[2] University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104

Received 24 March 2006/Accepted 23 May 2006

Haemophilus influenzae **requires either heme or a porphyrin and iron source for growth. Microarray studies of *H. influenzae* strain Rd KW20 identified 162 iron/heme-regulated genes, representing ~10% of the genome, with ≥1.5-fold changes in transcription in response to iron/heme availability in vitro. Eighty genes were preferentially expressed under iron/heme restriction; 82 genes were preferentially expressed under iron/heme-replete conditions.**

While *Haemophilus influenzae* is unable to synthesize protoporphyrin IX (PPIX), it does possess a ferrochelatase that reversibly inserts iron into PPIX to form heme (13, 23). Thus, *H. influenzae* requires either iron, in the presence of PPIX, or heme to grow. Found only in humans, *H. influenzae* has evolved highly redundant mechanisms to obtain both heme and iron from host sources (16). The available heme/iron sources and the *H. influenzae* acquisition mechanisms have been partially characterized (6, 7, 14, 16–20, 26), although little is known about the specific regulation of these pathways.

The goals of the current study were to identify gene products potentially mediating iron and heme uptake and to provide a foundation for their further study, as well as to gain insight into possible mechanisms of regulation. Since *H. influenzae* can interconvert heme and iron (releasing PPIX), the genomic transcriptional profiles of growth in iron- and heme (FeHm)-replete and -restricted media were compared.

Initially the kinetics of FeHm-mediated derepression were characterized using quantitative reverse transcription-PCR (Q-RT-PCR) to examine transcripts of the FeHm-regulated iron binding protein gene *hitA* (1, 16). Inocula of *H. influenzae* Rd KW20 (ATCC 51907) were prepared as previously described (14). Inocula were added to 60-ml volumes of brain heart infusion (BHI) broth containing 10 μg/ml β-NAD (BHI-NAD) to yield ~2×10^7 CFU/ml. FeHm-replete cultures (containing 500 μM $FeCl_3$ and 10 μg/ml heme as hemin chloride) and FeHm-restricted cultures (containing 150 μM deferoxamine) were incubated at 37°C in a rotary shaker (175 rpm). Samples were removed at 30-min intervals for Q-RT-PCR and viable count determination as previously described (15, 27). Cells remained viable upon transfer to FeHm-restricted BHI-NAD for at least 3 h (Fig. 1A), and transcription of *hitA* increased to a plateau within 60 to 90 min of transfer to an FeHm-restricted environment (Fig. 1B). Conversely, transcription of *hitA* in FeHm-supplemented media showed no increase over the same period.

To determine the kinetics of FeHm repression, the experiment was repeated with supplementation of the FeHm-restricted media at 90 min with 500 μM $FeCl_3$ and 10 μg/ml heme. Q-RT-PCR analysis demonstrated an initial increase in *hitA* transcripts; however, within 15 min of FeHm addition, transcripts rapidly decreased to a level identical to that in the FeHm-replete flask (Fig. 1C).

The transcription of *tbp1* (11) and *ompP2* (2) was also examined. These genes were considered to represent an iron/heme-regulated gene and a nonregulated gene, respectively. Genes *hitA* and *tbp1* share a similar transcriptional profile, while *ompP2* showed no changes in transcription (Fig. 1D). Oligonucleotide primers are listed in Table S1 in the supplemental data at http://peds.ouhsc.edu/lab/stull/whitby2006/tableS1.pdf.

The kinetics of Fe repression and heme repression are similar. The above experiment was repeated with addition of iron or heme alone. There were no apparent differences after supplementation with either iron or heme alone or with both in repression of transcription of *hitA* or *tbp2* (Fig. 2); therefore, dual-iron/heme supplementation was utilized in further studies.

Identification of the FeHm regulon of *H. influenzae*. The FeHm-restricted and FeHm-supplemented samples for microarray analysis were taken from a single flask, separated by 20 min. Three biological replicates of each condition were examined. The first sample was removed from FeHm-restricted media at 90 min postinoculation. Immediately, the flask was FeHm supplemented and a second sample was removed 20 min later. RNA for the microarray was prepared using Trizol (Invitrogen) as described by the manufacturer. Processing of the *H. influenzae* microarray was performed by Nimblegen (Madison, Wis.) following their protocols. Technical array replicates (arising from the presence of three arrays on each slide) were averaged prior to analysis of the biological replicates (8). The data were $\log_2$ transformed and compared between the two conditions by performing individual *t* tests using the TMEV software at http://www.tm4.org/mev.html (22). Genes with a 1.5-fold expression change and $P < 0.05$ were considered significant.

Of the 1,657 open reading frames on the Rd KW20 array, 162 were significantly differentially expressed (see Table S2 in the supplemental material at http://peds.ouhsc.edu/lab/stull/whitby2006/tableS2.pdf): 82 were maximally expressed under FeHm-replete conditions, and 80 were preferentially expressed under FeHm-restricted conditions. Comparison of microarray

* Corresponding author. Mailing address: Department of Pediatrics, CHO 2308, 940 NE 13th St., Oklahoma City, OK 73104. Phone: (405) 271-4401. Fax: (405) 271-8710. E-mail: tstull@ouhsc.edu.

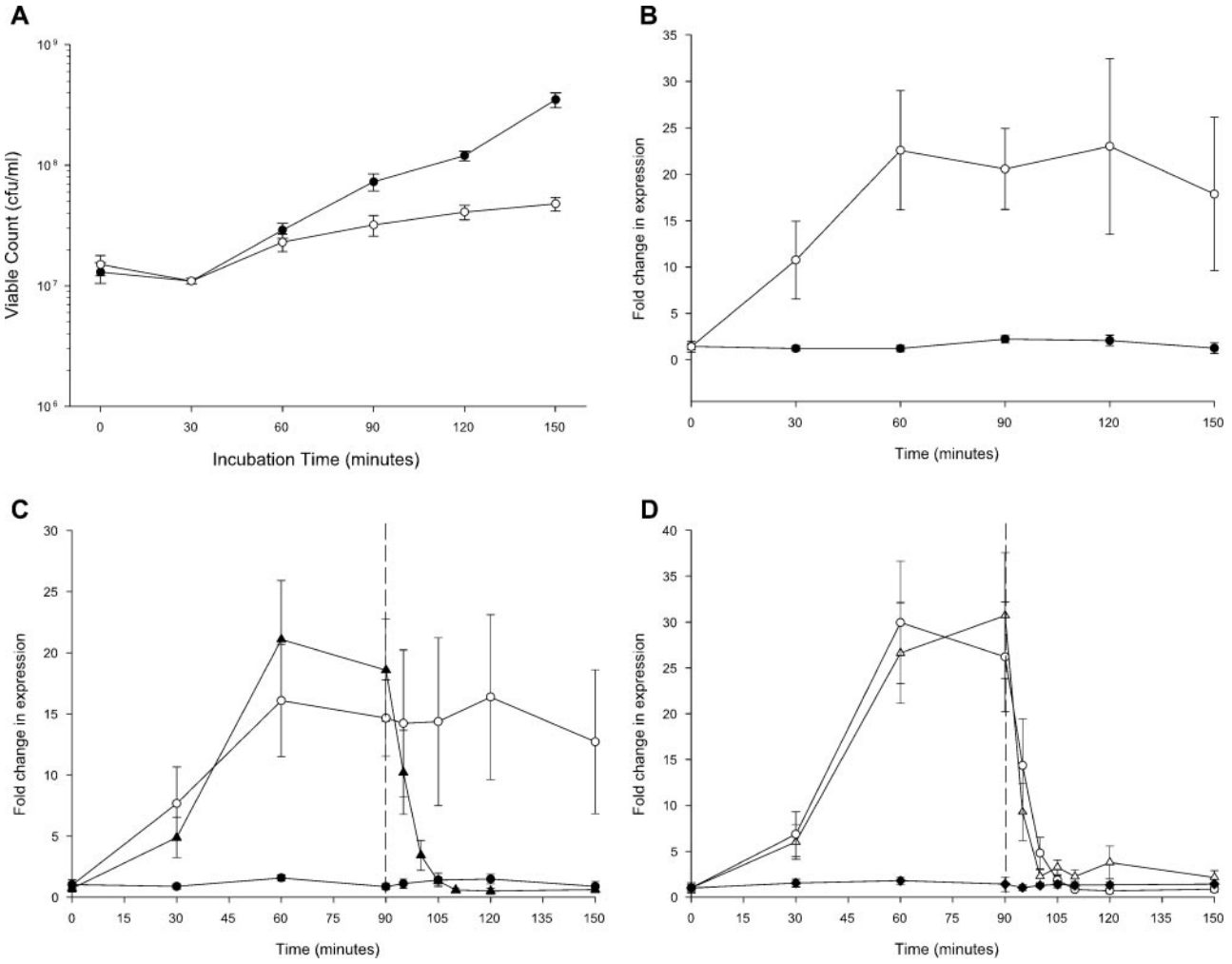

FIG. 1. Derepression and repression kinetics defining the window of transcriptional regulation for select FeHm-regulated genes of *H. influenzae* Rd KW20. (A) Growth kinetics (viable count) for the FeHm-replete (closed circles) and FeHm-restricted (open circles) media. (B) Transcription of the *hitA* gene in FeHm-replete (closed circles) and FeHm-restricted (open circles) media. (C) Comparison of transcription of the *hitA* gene in medium that was FeHm replete (closed circles), FeHm restricted (open circles), or initially FeHm restricted but to which FeHm was added at 90 min (closed triangles). (D) Comparison of transcription of the genes *hitA* (open circles), *tbp1* (open triangles), and *ompP2* (closed diamonds) in medium that was initially FeHm restricted but to which FeHm was added at 90 min. The vertical dashed line indicates the point at which FeHm was added.

and Q-RT-PCR data for 11 genes indicated a linear correlation, with the microarray underestimating Q-RT-PCR values by approximately threefold.

FeHm-repressed transcripts contain genes with a known role in iron/heme uptake. Of the 80 genes identified, 29 were single genes and 51 were in 21 putative operons. Analysis of the other genes in each operon indicated that while some genes had an increase in expression below the 1.5-fold threshold, all showed a statistically significant increase. Operons and standalone genes were determined based on a number of factors, including comparative genomics, transcriptional data from previous studies, conformity of orientation, size of intergenic regions, and prediction of putative transcriptional terminators using the program Transterm at http://cbcb.umb.edu/software/transterm (10). Included among the FeHm-repressible genes were known iron and heme acquisition genes (Table 1) including the hemoglobin/hemoglobin-haptoglobin receptor gene *hgpC*. (Strain Rd KW20 contains three potentially functional *hgp* genes, *hgpBCD*; however, both *hgpB* and *hgpD* are out of frame and not expressed.) To gain insight into possible mechanisms of regulation, we examined the upstream DNA for the presence of sequences associated with regulation by the ferric uptake regulator Fur (25). Potential sites were identified upstream of 32% of the FeHm-repressible operons.

Identification of other genes with a putative role in iron/heme uptake. Several additional genes encoding proteins with putative roles in iron transport were identified, including *afuABC* and *yfeABCD* and locus tags HI0663, HI0664, and HI1427. The *afu* locus shares homology with an *Actinobacillus pleuropneumoniae* ABC iron transporter (5). While the functions of HI1427, HI0663, and HI0664 are unknown, the putative product of HI1427 has characteristics consistent with periplasmic binding proteins, while the products of HI0663 and HI0664 both have characteristics of fused permease-ATPase

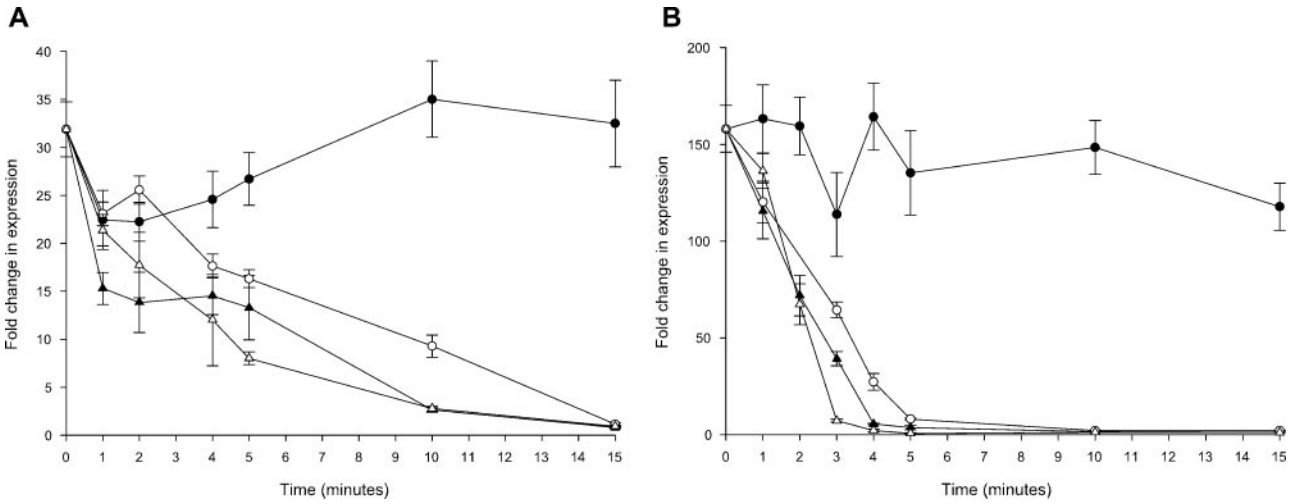

FIG. 2. Comparison of the transcription change of the genes *hitA* (A) and *tbp2* (B) following addition of iron (open circles), heme (closed triangles), or iron and heme (FeHm) together (open triangles) or with no addition (closed circles) following growth in FeHm-restricted medium. The abscissa values are time postsupplementation of the culture medium and correspond to the region following the dotted line shown in Fig. 1C and D.

components of ABC transporters. The *yfe* locus has been shown to mediate uptake of iron, manganese, and zinc in *Yersinia pestis* (3). Interestingly, transcription of a putative protease gene, *pqqL* (HI1368), was repressed by addition of FeHm. *pqqL* is in an operon encoding the TonB-dependent protein HI1369. Preliminary data indicate that HI1369 has a role in heme acquisition (data not shown). In other bacterial species, proteases play a direct role in the acquisition of iron and heme from various sources (12, 21, 24, 28).

The FeHm regulon contains genes with no known role in iron/heme uptake. A number of genes preferentially expressed under FeHm limitation appear to have a direct role on cellular metabolism. These include genes encoding the enzymes phosphoenolpyruvate carboxykinase and fructose-1,6- bisphosphatase, suggesting that there may be a cellular shift to gluconeogenesis in the absence of FeHm. Consistent with this are the observations that genes encoding aspartate ammonia lyase, asparaginase B, malate dehydrogenase, and fumerase C are repressed by FeHm addition. Together, these enzymes would lead to the conversion of L-asparagine to phosphoenolpyruvate, which then feeds into gluconeogensis to generate glucose phosphate. The latter compound may then be shunted into glycogen by the action of the products of the *malQ-glg* locus, also preferentially expressed under FeHm limitation.

Identification of FeHm-induced genes. Eighty-two genes were preferentially expressed under FeHm-replete conditions: of these 17 were stand-alone genes and 65 were contained within 25 putative operons (Table 1). Several of the identified genes have a role in iron and heme processing. For example, increased transcription of the bacterial ferritin genes (*ftnA1* and *ftnA2*) would increase the ability to store and thus detoxify excess iron. Similarly, transcription of *ccmDEFG* (encoding a heme exporter involved in the biosynthesis of cytochrome *c*) is increased. Genes having a potential role in respiration display increased transcription. These include *cydAB*, encoding subunits of the terminal electron acceptor cytochrome *d* ubiquinol oxidase, and the *nqr* operon encoding an Na^+-translocating NADH quinone oxidoreductase. This operon is similarly upregulated in Fe-rich environments in *Neisseria gonorrhoeae* (9). Other operons contain genes with iron-sulfur clusters (*fdxH*) or heme (*fdxI*) as cofactors.

Q-PCR of genes not represented on the microarray. Two operons, which were repressed by FeHm, lacked several of the predicted genes on the array. HI0007 to HI0009 were shown, by microarray, to be upregulated upon the addition of FeHm. The genes represented by these three locus tags form a putative operon with HI0006m and together encode the subunits of the nitrate-inducible formate dehydrogenase. HI0006m was considered a pseudogene due to an internal stop codon and was not included on the microarray design. However, this stop codon is conserved in other bacteria, encoding a selenocysteine residue in the *E. coli* HI0006m homolog (4). Comparison of RNA levels of HI0006m and HI0007 using Q-PCR confirmed that HI0006m is also repressed by addition of FeHm, consistent with the other three genes of this operon.

The transcription of HI0343 and HI0345 (both excluded from the array) was compared to levels of HI0344. This analysis determined that the genes represented by these two locus tags are also upregulated by the addition of FeHm, consistent with the other genes in that operon (data not shown).

Conclusion. The purpose of this study was to identify *H. influenzae* genes affected by iron and heme uptake. Defining the regulatory kinetics characterized a minimum window most likely to represent FeHm effects. Genes known to be involved in iron and heme uptake were clearly regulated in a synchronous manner. Furthermore, significant changes in transcription of all genes in the regulated operons were demonstrated. The identification of FeHm-regulated protease and potential periplasmic transport systems provides targets for future studies. Since iron and heme are sequestered in vivo, it is likely that the pattern of regulation observed in vitro relates to the transcriptional status during infection, supporting previous studies demonstrating transcription of FeHm-repressible genes during otitis media (29).

TABLE 1. *H. influenzae* Rd KW20 operons with altered transcription in response to environmental iron/heme levels

Operon locus[a]	No. of genes in operon[b]	Gene(s)[c]	Fold change[d]		Fur box[e]	Description[f]
			Maximum	Minimum		
FeHm repressible						
HI0017	1	*yfiD*	−1.87		Yes	Putative formate transferase
HI0020←HI0025	6		−1.75	−1.20		Citrate utilization operon
HI0035	1	*yidE*	−1.55			Putative permease
HI0075	1	*nrdD*	−4.03			Anaerobic ribonucleoside triphosphate reductase
HI0091←HI0092	2	*garK*	−1.84	−2.04		Probable glycerate kinase and probable gluconate family permease
HI0095	1		−10.21		Yes	Putative methyltransferase
HI0097→HI0099	3	*hitABC*	−8.15	−3.55	Yes	ABC transporter, transport of iron across cytoplasmic membrane
HI0113	1		−2.36		Yes	Probable TonB-dependent protein involved in iron/heme uptake
HI0126←HI0131	3	*afuCBA*	−1.90	−1.62	Yes	ABC transporter, *A. pleuropneumoniae* homolog involved in iron uptake
HI0205←HI0206	2	*nucA*	−1.60	−1.56		HI0205 has no predicted function; *nucA* is involved in NAD uptake
HI0213	1		−1.68		Yes	Putative component of ABC-type oligopeptide transport system
HI0223	1		−2.45		Yes	Predicted RarD family permease
HI0251→HI0253	3	*tonB*, *exbD*, *exbB*	−3.02	−2.69		Energy transduction for heme and iron transport
HI0257	1	*yfiA*	−1.61			Protects ribosomes during stationary-phase growth
HI0262→HI0264	3	*hxuCBA*	−10.30	−7.44	Yes	Heme acquisition from hemopexin
HI0359←HI0362	4	*yfeDCBA*	−11.75	−4.35	Yes	ABC transporter; *Y. pestis* homologs involved in Fe, Mn, and Zn uptake
HI0371←HI0380	10	*iscX*, *fdx-1*, *iscR*	−1.88	−1.22		Probable involvement in Fe-S cluster formation
HI0404←HI0405	2	*tilS*, *pdxY*	−1.88	−1.46		Pyroxidine kinase (*pdxY*) and tRNA modification (*tilS*)
HI0501→HI0507	6	*rbsGACBK*	−2.50	−1.23		Ribose transport
HI0534	1	*aspA*	−3.12			Conversion of aspartate to fumarate
HI0584	1		−1.69			Putative hydrolase/peptidase
HI0588	1		−1.60			Putative hydrolase/peptidase
HI0590←HI0591	2	*potE*, *speF*	−3.84	−3.20		Uptake of putrescine (*potE*) and ornithine decarboxylase (*speF*)
HI0601	1	*tfoX*	−1.66			Involved in regulation of competence
HI0663→HI0664	2	*cydDC*	−1.61	−1.61	Yes	Probable ABC transporter
HI0669→HI0670	2	*mioC*, *dtd*	−1.67	−1.60	Yes	Flavodoxin (*mioC*) and D-amino acid/tRNA deacylation (*dtd*)
HI0688→HI0689	2	*glpQ*	−1.53	−1.30		Glycerophosphoryl diester phosphodiesterase (*glpQ*)
HI0690→HI0691	2	*glpK*	−1.61	−1.44		Glycerol uptake facilitator (*glpF*) and glycerol kinase (*glpK*)
HI0712	1	*hgpC*	−6.64		Yes	Acquisition of heme from hemoglobin and hemoglobin-haptoglobin
HI0745	1	*ansB*	−3.42			Periplasmic asparaginase
HI0809	1	*pckA*	−2.87			Phosphoenolpyruvate carboxykinase, involved in gluconeogenesis
HI0836	1	*genX*	−1.61			Putative tRNA synthetase
HI0863	1	*pdxH*	−1.51			Pyridoxamine phosphate oxidase
HI0990	1	*iga1*	−1.52		Yes	Immunoglobulin A protease
HI0994←HI0995	2	*tbp1*, *tbp2*	−13.94	−12.40	Yes	Acquisition of iron from exogenous transferrin
HI1189←HI1191	3		−1.67	−1.67		Conserved hypothetical proteins
HI1210	1	*mdh*	−2.47			Malate dehydrogenase; role in respiration, fermentation, and gluconeogenesis
HI1217	1	*hup*	−2.99		Yes	Involved in uptake of heme from various sources
HI1227	1	*uraA*	−1.72			Uracil uptake
HI1228	1	*upp*	−1.62			Phosphorylation of uracil to UMP
HI1275	1	*tehB*	−3.34			Tellurite resistance
HI1349	1		−1.53			Bacterial ferritin-like protein
HI1356→HI1360	5	*malQ*, *glgBXCA*	−1.82	−1.51		Glycogen biosynthesis
HI1368→HI1369	2	*pqqL*	−7.02	−1.86	Yes	Putative protease (*pqqL*) and probable TonB-dependent transporter (HI1369)
HI1398	1	*fumC*	−2.32			Fumarate hydratase, reversible conversion of malate to fumarate
HI1427	1		−6.12			Putative ABC transporter, periplasmic binding protein
HI1564	1		−2.04			Probable pseudogene
HI1625←HI1626	2		−1.72	−1.38		Conserved hypothetical proteins
HI1645	1	*fbp*	−1.54			Fructose-1,6-bisphosphatase, involved in gluconeogenesis
HI1660	1	*nrdB*	−1.54			Ribonucleoside phosphate reductase
FeHm inducible						
HI0006→HI0009	4	*fdxHIE*	3.40	1.88		Formate dehydrogenase-N, involved in anaerobic respiration
HI0157	1	*fabH*	1.55			β-Ketoacyl-acyl carrier protein synthase III, fatty acid biosynthesis
HI0164→HI0171	6	*nqrABCDE6*	1.63	1.53		Ubiquinone reduction and energy metabolism
HI0172→HI0174	3	*apbE*, *mnmA*	1.93	1.69		Putative Fe-S cluster metabolism (*apbE*) + tRNA (5-methyl aminomethyl-2-thiouridylate)-methyltransferase (*mnmA*)
HI0184←HI0185	2	*adhC*	8.04	7.93		Putative esterase (HI0184), alcohol dehydrogenase (*adhC*)
HI0230	1	*nlpI*	2.30			Conserved lipoprotein of unknown function
HI0231	1	*deaD*	2.48			RNA helicase involved in rearrangement of 50S ribosome
HI0242←HI0244	3	*tgt*	2.30	1.58		Biosynthesis of queuine in tRNA wobble positions (*tgt*)
HI0318→HI0319	2	*yecO*	1.96	1.42		Probable methyltransferases
HI0321→HI0322	2	*vapB1*, *vapC1*	1.64	1.64		Probable toxin-antitoxin locus
HI0324	1	*rnt*	1.63			tRNA turnover and rRNA maturation
HI0343→HI0348	7	*napDGHBC*	1.93	1.66		Periplasmic nitrate reductase subunits
HI0381←HI0386	6	*tolR*, *ybgC*	1.52	1.33		Tol-Pal system, probable transport system from outer membrane to cytoplasm

Continued on following page

TABLE 1—*Continued*

Operon locus[a]	No. of genes in operon[b]	Gene(s)[c]	Fold change[d]		Fur box[e]	Description[f]
			Maximum	Minimum		
HI0419	1		1.58			Putative protease
HI0507	1		2.04			Conserved hypothetical protein
HI0512←HI0513	2	*hindIIR*, *hindIIIM*	1.65	1.53		Type 2 restriction endonuclease and methylase (HindII)
HI0623→HI0625	3	*fmt*, *rrmB*	1.62	1.44		tRNA modification (*fmt*), rRNA modification (*rrmB*), K^+ uptake (*trkA*)
HI0682	1	*ilvC*	1.55			Ketol-acid reductoisomerase, involved in isoleucine and valine biosynthesis
HI0864	1	*bipA*	1.71			Translation factor for Fis (HI0980)
HI0877←HI0878	2	*cgtA*	1.96	1.32		Putative ribosome-associated GTPase (*cgtA*)
HI0976→HI0978	3	*prmA*	1.54	1.29		Methyltransferase for 50S ribosomal protein L11 (*prmA*)
HI0979→HI0980	2	*dusB*, *fis*	2.06	2.00		tRNA-dihydrouridine synthase (*dusB*), DNA architecture modification (*fis*)
HI0998→HI1001	4	*rpL34*, *rnpA*, *yidC*	1.70	1.24		Ribosomal protein (*rpL34*), protein component of RNase P (*rnpA*); hypothetical protein (HI1000), inner membrane protein translocase (*yidC*)
HI1002	1	*trmE*	1.72			GTPase involved in biosynthesis of hypermodified nucleosides in tRNA
HI1005	1		1.64			Conserved hypothetical protein
HI1010→HI1016	7		1.86	1.47		Operon contains putative transport proteins and enzymes of unknown function
HI1037	1		1.81			Conserved hypothetical protein
HI1051	1	*vcaM*	1.80			Probable multidrug resistance ABC transporter
HI1066←HI1069	4	*nrfABCD*	2.13	1.52	Yes	Probable nitrite reductase
HI1075←HI1076	2	*cydBA*	1.72	1.63		Cytochrome *d* ubiquinol oxidase subunits, involved in respiration
HI1078←HI1080	3		1.62	1.22		ABC transport system, possibly for an amino acid (by homology)
HI1089→HI1097m	9	*ccmDEFG*	1.81	1.30		Heme exporter and cytochrome *c* biogenesis machinery
HI1173	1	*sprT*	1.52		Yes	Conserved hypothetical protein
HI1214→HI1216	3	*recJ*, *pfs*	1.74	1.43		Single-stranded DNase (*recJ*); 5′-methylthioadenosine/*s*-adenosylhomocysteine nucleosidase (*pfs*)
HI1253→HI1254	2		1.76	1.65		Conserved hypothetical proteins
HI1282→HI1284	3	*yhbC*, *nusA*, *infB*	1.96	1.53		Modulator of transcription termination (*nusA*), essential element of translation (*infB*)
HI1384→HI1385	2	*ftnA1*, *ftnA2*	2.94	2.60	Yes	Ferritin, iron storage proteins
HI1386	1		3.28			Putative glycosyltransferase
HI1584←HI1585	2	*ilvHI*	2.49	2.36		Acetolactate synthase III, valine biosynthesis
HI1586	1		1.56		Yes	Conserved hypothetical protein
HI1706	1	*betT*	3.14			Involved in osmoprotection
HI1733	1	*rnb*	1.81			RNase II, modulation of RNA decay

[a] Operon locus based on the Rd KW20 gene designation. Each arrow indicates the direction of the operon. The locus tag with the lowest number in the operon is given first for ease of reference.
[b] Number of genes comprising the putative operon. Note that HI0006, HI0343, and HI0345 were not included on the array.
[c] Genes contained within the operon that have been functionally characterized or identified by significant homology to other characterized genes.
[d] Change as determined from the microarray data. Shown are the maximum and minimum values observed for genes in the putative operon.
[e] "Yes" indicates identification of a putative Fur box based on homology to Fur consensus sequences.
[f] Brief description of the assigned functions of gene products encoded in the operon.

Microarray data accession number. Microarray data have been deposited with the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession no. GSE5061.

This work was supported by Public Health Service grant AI29611 from the National Institutes of Allergy and Infectious Diseases to T.L.S., D.J.M., and P.W.W. We gratefully acknowledge the support of the Children's Medical Research Institute.

We thank Jennifer Springer for technical assistance.

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