Published in final edited form as:

Microb Pathog. 2011 September ; 51(3): 133–141. doi:10.1016/j.micpath.2011.04.009.

The cyclic-AMP receptor protein (CRP) regulon in *Aggregatibacter actinomycetemcomitans* includes leukotoxin

Leigh A. Feuerbacher, Alex Burgum, and David Kolodrubetz*

Department of Microbiology and Immunology, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX, 78229-3900 USA

Abstract

The cyclic-AMP receptor protein (CRP) acts as a global regulatory protein among bacteria. Here, the CRP regulon has been defined in *Aggregatibacter actinomycetemcomitans* using microarray analysis of *A. actinomycetemcomitans* strain JP2 wild type cells compared to an isogenic *crp* deletion mutant. Genes whose expression levels changed at least 2-fold with $p \leq 0.05$ were considered significant. Of the 300 genes identified as being CRP-regulated, 139 were CRP-activated, including leukotoxin, with the remaining being CRP-repressed. The 300 genes represent 14.2% of ORFs probed which is significantly higher than what has been reported for CRP regulons in other bacteria. If the CRP-regulated genes are put into 17 functional classes, all 17 categories had at least 1 CRP-regulated gene. Several functional categories, mainly transport and binding proteins and energy metabolism proteins, were disproportionately represented in the CRP-regulated subset of genes relative to their overall representation in the genome. This is similar to the patterns seen in other bacteria. Finally, quantitative RT-PCR was used to show that the leukotoxin RNA levels were repressed 16-fold in the CRP mutant indicating that CRP activates leukotoxin transcription. However, this regulation appears to be acting through another regulatory protein since the leukotoxin promoter, unlike ~129 other promoters of CRP-regulated genes, does not have a match to the consensus CRP binding site. Several candidate genes for this intermediary transcription factor have been identified in the CRP-regulon.

Keywords

Aggregatibacter actinomycetemcomitans; cyclic-AMP receptor protein; CRP regulon; leukotoxin

1. Introduction

The gram-negative capnophilic bacterium *Aggregatibacter actinomycetemcomitans* is a bacterial resident of the oral cavity that has been implicated in adult periodontal disease as well as in non-oral infections, such as endocarditis [1–4]. However, this bacterium is most often associated with localized aggressive periodontitis (LAP) [2,5–7]. LAP has an early onset in life, typically afflicting adolescents. The disease is usually localized to the first

© 2011 Elsevier Ltd. All rights reserved.

*Correspondence: Dr. David Kolodrubetz, Department of Microbiology and Immunology, Mail Code 7758, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229, USA, Tel.: +1 210 567 3967; Fax: +1 210 567 6612; kolodrubetz@uthscsa.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

molars and central incisors and is characterized by rapid tissue and alveolar bone destruction and it may ultimately lead to tooth loss if left untreated [8,9].

A. actinomycetemcomitans expresses a number of potential virulence factors in order to initiate and cause disease [8, 10, 11]. The most studied virulence factor is the 116-kDa leukotoxin [12, 13], in part because strains which express high levels of leukotoxin are most often associated with disease [14]. This protein is thought to help *A. actinomycetemcomitans* avoid host cell defenses by targeting and killing human polymorphonuclear leukocytes, macrophages, [15–18] and erythrocytes [19], although it may induce apoptosis of cells at lower concentrations [20]. Interestingly, leukotoxin-induced killing of macrophages proceeds through a novel mechanism that leads to the release of pro-inflammatory cytokines during macrophage cell death [21, 22]. Early on, it became clear that the various strains of *A. actinomycetemcomitans* could be broadly placed into two groups based upon their levels of leukotoxin; highly leukotoxic strains make 10–20 times as much leukotoxin as moderate/low leukotoxin-producing strains [23]. The difference in leukotoxin expression between these groups is not due to sequence changes in their leukotoxin promoter regions, but is, instead, due to a deletion of 528 bp in the 5'-non-coding region of the leukotoxin operon [24, 25]. The mechanism by which this difference in the mRNA leader between strains leads to altered leukotoxin RNA levels is not known. Not surprisingly, this virulence factor is also regulated by a number of different environmental conditions. For example, anaerobiosis results in the induction of leukotoxin transcription and protein synthesis [26, 27]. Fong *et al.* reported that the secreted AI2 product of the *luxS* gene, a gene involved in quorum sensing, induced leukotoxin activity 3-fold [28]. The localization of leukotoxin, but not its transcription, is influenced by iron concentration; the protein is cell-surface-associated in iron-rich media but most of the leukotoxin protein is secreted into the media if iron is limiting [29]. More recently, *ptsH* and *ptsI*, which encode proteins of the phosphotransferase system, were shown to be required for maximal leukotoxin RNA synthesis [30]. Several years ago, Mizoguchi *et al.* and Inoue *et al.* found that leukotoxin transcription is affected by carbon source; limiting concentrations of fermentable sugars increase leukotoxin production. In addition, they showed that cyclic-AMP (cAMP) levels were proportional to leukotoxin levels [31, 32]. Since, in *E. coli*, cAMP binds to the cAMP receptor protein (CRP) [33] which regulates transcription at promoters containing reasonable matches to the consensus sequence, TGTGA-N₆-TCACA, it is reasonable to suggest that leukotoxin in *A. actinomycetemcomitans* may be regulated by catabolite repression and CRP.

However, sequence inspection indicates that there is no CRP binding site within the *A. actinomycetemcomitans* leukotoxin promoter, which raises the question of whether or not CRP is involved in the regulation of leukotoxin synthesis and, if so, how. To determine if CRP does indeed alter leukotoxin transcription in *A. actinomycetemcomitans*, we have constructed and characterized a *crp* mutant. Examination of leukotoxin protein and RNA production in wild type and mutant cells shows that CRP is required for leukotoxin transcription. To identify other CRP-regulated genes in *A. actinomycetemcomitans*, whole genome microarray analyses were done with RNA from wild type and *crp* mutant cells. The results indicate that hundreds of genes are CRP-regulated in *A. actinomycetemcomitans* and that its CRP appears to bind to the same sequences as the *E. coli* protein. In addition, there are several CRP-regulated transcription factors; these proteins are candidates for the molecules that CRP uses to regulate leukotoxin transcription.

2. Results

2.1 CRP is involved in leukotoxin protein and RNA synthesis

Although there are several reports suggesting that cyclic-AMP receptor protein (CRP) is involved in controlling leukotoxin synthesis in *A. actinomycetemcomitans* [31, 32], this

hypothesis has not been directly tested. In order to prove that CRP is, or is not, involved in leukotoxin production, we identified a CRP ortholog in *A. actinomycetemcomitans* and constructed a *crp* deletion mutant in *A. actinomycetemcomitans* strain JP2 by allelic replacement. Wild type and *crp* mutant cells were grown anaerobically, in the presence of excess FeCl_3 to suppress leukotoxin secretion into the media [29], and protein from whole cell lysates was analyzed by SDS-PAGE and Western blot analysis for leukotoxin production. Both experiments show that the *crp* mutant produces significantly less leukotoxin than wild type cells (Fig. 1). Importantly, complementation of the *crp* deletion with a plasmid containing only the wild type *crp* gene restored leukotoxin protein expression (Fig. 1) indicating that it is CRP that is involved in regulating leukotoxin production.

To see if the activation of leukotoxin expression by CRP was occurring at the level of transcription, RNA was isolated from wild type and *crp* mutant cells and analyzed by quantitative reverse transcriptase PCR. Consistent with the protein results, the *crp* mutant had a significant reduction (16.1, $p \leq 0.05$ by Student's t-test) in *ltxA* RNA levels (Fig. 2). This indicates that CRP is affecting leukotoxin RNA expression, most likely at the transcriptional level.

2.2 Global gene expression profiling of the *crp* mutant

In other bacteria, CRP is a global regulator of hundreds of genes [34–38]. To see if CRP is a global regulator in *A. actinomycetemcomitans* and to characterize its regulon, we compared the gene expression profiles of the *crp* deletion mutant to its wild type parent, JP2. Three hundred genes had a significant ($p \leq 0.05$) change in expression of 2-fold or greater in the *crp* mutant (Tables S1 and S2). This number represents 14.2% of the genes probed during microarray analysis. Slightly less than half, 139, of the CRP-regulated genes are activated by CRP (Table S1). The rest (161) are repressed by CRP (Table S2). The 300 CRP-regulated genes appear to be organized into 177 operons, fifty-nine of which contain two to nine genes each. As expected from our real time-RT-PCR results with *ltxA* in the CRP mutant, the four genes in the leukotoxin operon [24, 39] are included in the CRP-activated set (Table S1, operon 75).

When CRP-regulated *A. actinomycetemcomitans* genes are categorized into seventeen classes, based broadly upon their presumed function (Oral Pathogen Sequence Databases, Los Alamos National Laboratory), every functional category has at least one gene that is CRP-regulated (Tables S3 and S4). However, there are several functional classes whose genes are disproportionately regulated by CRP, relative to their overall representation in the genome (Fig. 3). Genes in the transport and binding proteins category are clearly over-represented among both the CRP-activated and CRP-repressed genes. This is not surprising since this category includes proteins that take different sugars into cells and these types of genes are classically regulated by catabolite repression and CRP [40]. The “other categories” functional class is also over-represented in CRP-activated genes. The CRP-regulated genes in this category mainly consist of phage-related transposase or integrase genes. The CRP-repressed genes have a disproportionate number of genes in the functional category of energy metabolism and amino acid biosynthesis. The latter are mainly involved in aromatic amino acid biosynthesis and the former largely encompass genes involved in anaerobic respiration and electron transport. Finally, the combination of hypothetical and hypothetical

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of genes, organized into operons, that are CRP-activated (≥ 2 -fold) in *A. actinomycetemcomitans*

Table S2. List of genes, organized into operons, that are CRP-repressed (≥ 2 -fold) in *A. actinomycetemcomitans*

Table S3. Genes significantly induced (≥ 2 -fold) by CRP in *A. actinomycetemcomitans*, organized by functional class

Table S4. Genes significantly repressed (≥ 2 -fold) by CRP in *A. actinomycetemcomitans*, organized by functional class

conserved genes contains the largest number of CRP-regulated genes, but they are, overall, actually slightly under-represented among the CRP-regulated genes when you consider that they constitute more than one-third of the total number of *A. actinomycetemcomitans* ORFs.

To identify the operons that are likely to be directly regulated by CRP, the sequences upstream of each of the 177 CRP-regulated operons were examined for potential CRP-binding sites [Patser score > 5.0 using the *E. coli* CRP binding matrix (Regulon DB)] [41]. If a potential CRP-binding site is in an intergenic region immediately upstream of an operon, it seems likely that the operon is directly regulated by CRP. As long as the putative CRP-binding site is in an intergenic region, its position relative to the translation start site was not used to exclude the CRP site as a candidate for direct activation, since it is the position of each CRP-binding site relative to the appropriate transcription start site that is critical and most of the transcription start sites have not been mapped in *A. actinomycetemcomitans*. Using these criteria, 37% of the CRP-activate operons (Table 1) and 36% of the CRP-repressed operons (Table 2) appear likely to be regulated directly by CRP. This represents a total of 129 genes that are likely to be regulated by CRP's interaction with their promoters. The genes that are directly regulated by CRP do not belong predominantly to any one or two functional classes (data not shown), but are distributed across seventeen functional categories with basically the same pattern seen for the entire set of CRP-regulated genes (Fig. 3).

Is an intergenic CRP-binding site a good prediction of CRP-regulation in our experiment? Apparently not. There are 77 intergenic CRP-binding sites (Patser score ≥ 10) in the entire *A. actinomycetemcomitans* genome. But only 19 (25%) of the genes adjacent to those CRP sites are CRP-regulated in our microarray experiment. This seemingly large percentage of CRP-binding sites that appear to be non-functional most likely represents the involvement of additional transcription factors at a lot of CRP-regulated genes under the conditions tested rather than the inability of these sites to bind CRP and alter transcription. This can be tested, in part, by additional expression array experiments with cells grown under different conditions where other transcription factors may play a less dominant role.

3. Discussion

Inoue *et al.* showed by Northern blot analysis that leukotoxin RNA levels decreased in response to increased fructose in the media [32]. Since this effect could be reversed by the addition of cAMP to the media, it was hypothesized, but not proven, that this regulation occurs via the classical cAMP/CRP regulatory pathway [33]. In this pathway, when glucose is present, the CRP protein is inactive and unable to bind DNA. However, when glucose is depleted cAMP is synthesized by the cell and it binds to and activates CRP. The active cAMP/CRP complex can then bind to its genomic binding sites and activate or repress the transcription of adjacent operons. In this report, we have clearly shown that CRP does regulate leukotoxin transcription since leukotoxin RNA levels decrease 16-fold in a CRP deletion mutant. Although our experiments were done in JP2, a highly leukotoxic strain, CRP will almost certainly activate leukotoxin transcription in other strains of *A. actinomycetemcomitans*, even those that have the 528 bp deletion in the 5'-non-coding region of the leukotoxin operon and express overall lower levels of leukotoxin, because (1) the strain, 301-b, Inoue et al [32] used to show that leukotoxin transcription is activated by cAMP (and thus presumably activated by CRP) has the 528 bp deletion in the leukotoxin mRNA leader but is identical to JP2 in the leukotoxin promoter region, and, (2) the leukotoxin promoter regions are identical among most strains of *A. actinomycetemcomitans* [24, 42, 43] or vary, in one strain, by a few bases [25]. However, the mechanism by which CRP activates leukotoxin transcription is unclear since the leukotoxin promoter does not have a sequence resembling the CRP-consensus binding sequence [33, 44]; the best match in

the leukotoxin promoter region has an extremely low Patser score of 3.7. This suggests that either cAMP/CRP is binding to a non-consensus sequence or it is acting indirectly at leukotoxin by regulating another transcription factor. Since the *A. actinomycetemcomitans* CRP protein sequence is 86% identical to that of the *E. coli* protein and since our microarray results show that a number of genes with close-to-consensus CRP-binding sites in their promoters are mis-regulated in our CRP mutant, we favor the latter possibility. In fact, eleven genes encoding possible DNA binding protein/transcription or regulatory factors are regulated by CRP in *A. actinomycetemcomitans*. We are currently investigating the possibility that one of these proteins may be the intermediary between CRP and its regulation of leukotoxin transcription.

In other bacteria, catabolite repression is a global regulatory mechanism that alters the transcription of numerous genes, especially those needed for utilization of non-glucose carbon sources [33, 45]. We have used microarray expression analysis to show that at least 14% of the genes in *A. actinomycetemcomitans* are CRP regulated either directly (6%) or indirectly (8%). This is significantly higher than what has been reported for other bacteria. In *Vibrio cholerae*, *Yersinia pestis* and *E. coli*, <7% of each organism's genes were CRP-regulated in microarray experiments [35, 37, 38]. The biological relevance of this difference is unclear since the growth parameters used with *V. cholerae* and *Y. pestis* were quite different and the *E. coli* results had the additional constraint that the CRP-regulated genes must also be glucose regulated. However, it is interesting to speculate that CRP regulates more genes in *A. actinomycetemcomitans* because, as the only oral organism examined, it needs to deal with more frequent changes in the carbohydrates seen than do the non-oral bacteria examined by microarrays. Despite the differences in the overall number of genes regulated by CRP in the different bacteria using different experimental parameters, the proportions of functional categories of genes that are CRP-regulated are very similar. For example, in *V. cholerae* and *A. actinomycetemcomitans*, which both used the same classification scheme to put genes into seventeen functional categories, the majority of CRP-regulated genes belong to one of three categories: transport/binding proteins, energy metabolism or hypothetical/hypothetical conserved (compare our Fig. 3 to Fig. 1 in reference [37]).

Sixty different genes appear to be regulated directly by CRP in *E. coli* [34–36] and/or *Y. pestis* [38]. However, only one of these genes, *ptsG*, appears to be regulated directly by CRP in all three organisms (*A. actinomycetemcomitans*, *E. coli*, and *Y. pestis*). In addition, the *malX* gene and *mglBAC* operon appear to be regulated directly by CRP in *E. coli* and *A. actinomycetemcomitans*. This result seems somewhat surprising since the overall classes of genes regulated by CRP seems so similar, but 35 of the 60 genes that are regulated directly by CRP in *E. coli* or *Y. pestis* do not have homologues in *A. actinomycetemcomitans*. Once again this likely reflects the different ecological niches in which these organisms are found. Of the 25 genes that do have *A. actinomycetemcomitans* homologues, 19 are not CRP-regulated in *A. actinomycetemcomitans*. Of course, the caveat “not CRP-regulated in the conditions tested” must be added, especially in light of the fact that 9 of the *A. actinomycetemcomitans* homologues of directly regulated *E. coli* or *Y. pestis* genes have a good CRP-binding site (Patser score > 8.0) in their promoter regions and thus may actually be regulated directly by CRP in different growth conditions. This possibility is being examined.

In summary, microarray analysis of a *crp* mutant was used to define the CRP regulon of *A. actinomycetemcomitans*. The data indicates that, while the percentage of CRP-regulated genes is higher in *A. actinomycetemcomitans* than other bacteria, the distribution of these genes among functional categories is similar to patterns seen in other bacteria. This study also proved that CRP regulates leukotoxin production at the transcriptional level although

this most likely occurs through some intermediary protein. We have identified candidate genes for this intermediary factor which are currently being investigated for their roles in leukotoxin regulation.

4. Materials and Methods

4.1 Bacterial strains and culture conditions

A. actinomycetemcomitans strain JP2 [23] was used in this study. JP2, a serotype b strain, was originally isolated from a young patient with periodontitis and it expresses high levels of leukotoxin due to a 528 bp deletion in the 5'-non-coding region of the leukotoxin operon [24, 25]. Cells were grown anaerobically (5% CO₂, 10% H₂, 85% N₂) in a Coy chamber (Coy Laboratory Products, Ann Arbor, MI) at 37°C with shaking in TSBYE (3% tryptic soy broth plus 0.6% yeast extract). The recombinant constructs were propagated in *E. coli* TB-1 at 37°C in Luria broth [46]. Where indicated, FeCl₃ was added to a final concentration of 300 µM, ampicillin was added to a final concentration of 100 µg/ml, spectinomycin to a final concentration of 100 µg/ml or chloramphenicol to a final concentration of 5 µg/ml.

4.2 Construction of a *crp* deletion mutant in *A. actinomycetemcomitans*

To construct a *crp* deletion mutant, PCR and standard recombinant DNA techniques were used to construct plasmid pDK936, in which 0.98 kb of DNA immediately upstream of *crp* and 0.94 kb of the DNA immediately downstream of *crp* flank a 1.1 kb spectinomycin resistance gene [47] in place of the *crp* coding region. The plasmid, which is pUC-based and does not replicate in *A. actinomycetemcomitans*, contains *sacB* as a counterselectable marker [48]. Plasmid pDK936 was electroporated into *A. actinomycetemcomitans* strain JP2. Spectinomycin resistant colonies, resulting from a single crossover event at the *crp* locus, were selected. By subsequently plating the spectinomycin resistant cells onto TSBYE plus 10% sucrose, cells in which a second recombination event occurred, such that the vector is removed from the chromosome along with the wild type *crp* gene, were selected. PCR was used to confirm that the genomic *crp* gene had been replaced by the spectinomycin resistance allele in these spectinomycin-resistant, sucrose-resistant colonies, and one isolate, AAM167, was used in subsequent experiments.

4.3 Complementation of the *crp* deletion mutant

Standard recombinant DNA techniques and PCR were used to construct plasmid pDK937, a *crp* complementing plasmid. This DNA contains 319 bp of the region immediately upstream of *crp*, the entire *crp* gene and 48 bp of the DNA immediately downstream of *crp*, all cloned into pDK937 which is a derivative of the integrating plasmid pJM102 [49] that contains a chloramphenicol resistance gene. This plasmid was electroporated into AAM167 and the resulting chloramphenicol resistant colonies were shown to result from a single recombination event at the *crp* locus by PCR analysis. One isolate, AAM168, was used in subsequent experiments.

4.4 Analysis of leukotoxin protein

Cells from log phase cultures, grown with 300 µM FeCl₃, of *A. actinomycetemcomitans* strains JP2, AAM167 and AAM168 were collected by centrifugation. Total protein was prepared by suspending equal numbers of cells in 50 µl 1× loading buffer (1% SDS, 10% glycerol, 6 mM Tris, pH 6.8, 5% β-mercaptoethanol, and 0.01% bromophenol blue) and heating at 100°C for 2 min. The samples were clarified by centrifuging 5 min and equal amounts of each sample were electrophoresed on duplicate 7.5% SDS-polyacrylamide gels. Protein was visualized on one gel using Coomassie brilliant blue. The proteins from the other gel were electrophoretically transferred to a nitrocellulose membrane (Hoefer, Inc,

Holliston, MA) using CAPS buffer (10 mM CAPS, 3.24 mM DTT, 1% methanol) for Western blot analysis. After blocking for 1 h with blocking solution, diluted (1:2000) rabbit polyclonal anti-leukotoxin antibody was added to the nitrocellulose filter for 2 h, followed by the addition of a 1:5000 dilution of goat-anti-rabbit IgG whole molecule Horseradish peroxidase (HRP) conjugate (Sigma, St. Louis, MO). HRP color developing reagent (Bio-Rad, Hercules, CA) was used to develop the Western.

4.5 Analysis of RNA expression

RNA was isolated from mid-log phase *A. actinomycetemcomitans* cells using RNeasy[®] Bacterial Reagent (Qiagen, Inc., Valencia, CA) and the RNeasy[®] Mini Kit (Qiagen). The manufacturer's instructions were followed with the following notes: 2.5 ml of cells were lysed with 10 mg/ml lysozyme (Sigma, St. Louis, MO) for 15 min. The RNase-free DNase Set (Qiagen) was used to remove DNA. Each RNA sample (400 ng) was converted into cDNA with the Omniscript[®] Reverse Transcriptase kit (Qiagen) and random hexamer primers (Qiagen). Real time PCR was performed in triplicate on 1/1600 dilutions of cDNAs with GoTaq[®] qPCR Master Mix (Promega, Madison, WI) and appropriate primers (300 nM for lkt516, lkt517, lkt519; 900 nM for lkt518). The reactions were monitored on a Chromo4 Real-Time PCR Detection System (Bio-Rad, Hercules, CA); an initial denaturation step of 2 min at 95°C was followed by 39 cycles of amplification (15 s at 95°C, 1 min at 63°C, read plate). A melting curve of the reaction products was done at the completion of the PCR to show that the expected PCR product was the only one amplified. The level of *ltxA* mRNA in a given sample was normalized to the level of *pdxY* mRNA in the same sample by calculating the difference in the averaged *ltxA* and *pdxY* threshold cycles (C_T) for that sample. The following primer pairs were used: lkt516 (5'-CGAGGGAAGGTTACCGATCTAC-3') and lkt517 (5'-TGCTGCCGATAATTCCTCTAC-3') amplify a 119 bp fragment from the middle of the leukotoxin gene and primers lkt518 (5'-CAAAAGCCTCCACATCGGAT-3') and lkt519 (5'-CGTTGGCGTGGGTGATTTAAC-3') amplify a 77 bp fragment from the *pdxY* gene. This gene is not regulated in multiple microarray experiments of cells grown under various conditions (data not shown).

For microarray analysis, 50 µg of each RNA sample was sent on dry ice to Roche NimbleGen (Madison, WI) where the RNA was made into Cy3-labeled cDNA using random hexamers and hybridized to a custom *A. actinomycetemcomitans* array. The microarrays, designed and synthesized by Roche NimbleGen, were based on the sequence and annotation data for *A. actinomycetemcomitans* strain HK1651 (Oral Pathogen Sequence Databases, Los Alamos National Laboratories). The arrays were scanned at Roche NimbleGen and the expression values were sent back to UTHSCSA. Each array had 60-mer probes for 2,116 genes and there were 11 different probes/gene for almost all (94%) of the ORFs. In addition, this entire probe set was replicated three times on each array and there were 4,426 random probes per array.

4.6 Statistical analysis

Robust Multi-Array Analysis (RMA) [50] in the NimbleScan Software Package (v2.5, Roche NimbleGen, Madison, WI) was used to do background corrections of expression data from individual arrays, to then do quantile normalization [51] across the arrays being compared and to reduce the contribution of outlier probes to the single expression value derived from multiple probes for a given gene on a given array. The corrected expression values from three biological replicates of JP2 were compared to those from three biological replicates of AAM167 by performing student t-tests on the log 2 transformed data using the Multi Experiment Viewer (<http://www.tm4.org/mev>) [52]. Genes whose expression levels changed by at least 2-fold with $p \leq 0.05$ were considered significant.

4.7 Accession number

The microarray data is available online at the National Center for Biotechnology Information Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/>) and is MIAME-compliant. Its accession number is GSE25459.

4.8 Assigning genes to operons

Two or more ORFs are considered to be in an operon if they are in the same orientation and are not separated by any intergenic regions longer than 50 bp in length. If an intergenic sequence between two same-orientation ORFs was longer than 50 bp, the Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html) and BPROM (<http://linux1.softberry.com/berry.phtml>) programs were used to identify potential σ^{70} promoters in the intergenic region. If both programs predicted that there was a correctly oriented σ^{70} promoter, then the downstream ORF was assigned to a new operon, instead of being designated part of an operon with the upstream gene.

Acknowledgments

This work was supported by the Public Health Service Grants T32-DE14318 and DE15625 from the National Institutes of Health.

References

1. Nørskov-Lauritsen N, Kilian M. Reclassification of *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, *Haemophilus paraphrophilus* and *Haemophilus segnis* as *Aggregatibacter actinomycetemcomitans* gen. nov., comb. nov., *Aggregatibacter aphrophilus* comb. nov. and *Aggregatibacter segnis* comb. nov., and emended description of *Aggregatibacter aphrophilus* to include V factor-dependent and V factor-independent isolates. *Int J Syst Evol Microbiol.* 2005; 56:2135–2146.
2. Slots J, Ting M. *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in human periodontal disease: occurrence and treatment. *Periodontol 2000.* 1999; 20:82–121. [PubMed: 10522224]
3. van Winkelhoff AJ, Slots J. *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in nonoral infections. *Periodontol 2000.* 1999; 20:122–135. [PubMed: 10522225]
4. Mitchell RG, Gillespie WA. Bacterial Endocarditis due to an actinobacillus. *J Clin Pathol.* 1964; 17:511–512. [PubMed: 14207783]
5. Slots J. The predominant cultivable organisms in juvenile periodontitis. *Scand J Dent Res.* 1976; 84:1–10. [PubMed: 1061986]
6. Zambon JJ. *Actinobacillus actinomycetemcomitans* in human periodontal disease. *J Clin Periodontol.* 1985; 12:1–20. [PubMed: 3882766]
7. Henderson B, Ward JM, Ready D. *Aggregatibacter (Actinobacillus) actinomycetemcomitans*: a triple A* periodontopathogen? *Periodontol 2000.* 2010; 54:78–105. [PubMed: 20712635]
8. Fine DH, Kaplan JB, Kachlany SC, Schreiner HC. How we got attached to *Actinobacillus actinomycetemcomitans*: a model for infectious diseases. *Periodontol 2000.* 2006; 42:114–157. [PubMed: 16930309]
9. Kinane DF, Mooney J, Ebersole JL. Humoral immune response to *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in periodontal disease. *Periodontol 2000.* 1999; 20:289–340. [PubMed: 10522229]
10. Fives-Taylor PM, Meyer DH, Mintz KP, Brissette C. Virulence factors of *Actinobacillus actinomycetemcomitans*. *Periodontol 2000.* 1999; 20:136–167. [PubMed: 10522226]
11. Henderson B, Nair SP, Ward JM, Wilson M. Molecular pathogenicity of the oral opportunistic pathogen *Actinobacillus actinomycetemcomitans*. *Annu Rev Microbiol.* 2003; 57:29–55. [PubMed: 14527274]

12. Tsai CC, McArthur WP, Baehni PC, Hammond BF, Taichman NS. Extraction and partial characterization of a leukotoxin from a plaque-derived gram-negative microorganism. *Infect Immun.* 1979; 25:427–439. [PubMed: 113347]
13. Kachlany SC. *Aggregatibacter actinomycetemcomitans* leukotoxin: from threat to therapy. *J Dent Res.* 2010; 89:561–570. [PubMed: 20200418]
14. Haubek D. The highly leukotoxic JP2 clone of *Aggregatibacter actinomycetemcomitans*: evolutionary aspects, epidemiology and etiological role in aggressive periodontitis. *Acta Pathologica, Microbiologica et Immunologica Scandinavica.* 2010; 118 Suppl 130:1–53.
15. Taichman NS, Wilton JM. Leukotoxicity of an extract from *Actinobacillus actinomycetemcomitans* for human gingival polymorphonuclear leukocytes. *Inflammation.* 1981; 5:1–12. [PubMed: 7228351]
16. Lally ET, Kieba IR, Sato A, Green CL, Rosenbloom J, Korostoff J, et al. RTX toxins recognize a $\beta 2$ integrin on the surface of human target cells. *J Biol Chem.* 1997; 272:30463–30469. [PubMed: 9374538]
17. Johansson A, Sandström G, Claesson R, Hånström L, Kalfas S. Anaerobic neutrophil-dependent killing of *Actinobacillus actinomycetemcomitans* in relation to the bacterial leukotoxicity. *Eur J Oral Sci.* 2000; 108:136–146. [PubMed: 10768727]
18. Venketaraman V, Lin AK, Le A, Kachlany SC, Connell ND, Kaplan JB. Both leukotoxin and poly-N-acetylglucosamine surface polysaccharide protect *Aggregatibacter actinomycetemcomitans* cells from macrophage killing. *Microb Pathog.* 2008; 45:173–180. [PubMed: 18573331]
19. Balashova NV, Crosby JA, Al Ghofaily L, Kachlany SC. Leukotoxin confers beta-hemolytic activity to *Actinobacillus actinomycetemcomitans*. *Infect Immun.* 2006; 74:2015–2021. [PubMed: 16552030]
20. Korostoff J, Wang JF, Kieba I, Miller M, Shenker BJ, Lally ET. *Actinobacillus actinomycetemcomitans* leukotoxin induces apoptosis in HL-60 cells. *Infect Immun.* 1998; 66:4474–4483. [PubMed: 9712803]
21. Kelk P, Claesson R, Chen C, Sjöstedt A, Johansson A. IL-1 β secretion induced by *Aggregatibacter (Actinobacillus) actinomycetemcomitans* is mainly caused by the leukotoxin. *Int J Med Microbiol.* 2008; 298:529–541. [PubMed: 17888725]
22. Kelk P, Abd H, Claesson R, Sandström G, Sjöstedt A, Johansson A. Cellular and molecular response of human macrophages exposed to *Aggregatibacter actinomycetemcomitans* leukotoxin. *Cell Death Dis.* 2011; 2:e126. [PubMed: 21390060]
23. Tsai CC, Shenker BJ, DiRienzo JM, Malamud D, Taichman NS. Extraction and isolation of a leukotoxin from *Actinobacillus actinomycetemcomitans* with polymyxin B. *Infect Immun.* 1984; 43:700–705. [PubMed: 6319288]
24. Brogan JM, Lally ET, Poulsen K, Kilian M, Demuth DR. Regulation of *Actinobacillus actinomycetemcomitans* leukotoxin expression: analysis of the promoter regions of leukotoxic and minimally leukotoxic strains. *Infect Immun.* 1994; 62:501–508. [PubMed: 8300209]
25. Kolodrubetz D, Spitznagel J Jr, Wang B, Phillips LH, Jacobs C, Kraig E. *cis* elements and *trans* factors are both important in strain-specific regulation of the leukotoxin gene in *Actinobacillus actinomycetemcomitans*. *Infect Immun.* 1996; 64:3451–3460. [PubMed: 8751884]
26. Spitznagel J Jr, Kraig E, Kolodrubetz D. The regulation of leukotoxin production in *Actinobacillus actinomycetemcomitans* strain JP2. *Adv Dent Res.* 1995; 9:48–54. [PubMed: 7669214]
27. Kolodrubetz D, Phillips L, Jacobs C, Burgum A, Kraig E. Anaerobic regulation of *Actinobacillus actinomycetemcomitans* leukotoxin transcription is ArcA/FnrA-independent and requires a novel promoter element. *Res Microbiol.* 2003; 154:645–653. [PubMed: 14596902]
28. Fong KP, Chung WO, Lamont RJ, Demuth DR. Intra- and interspecies regulation of gene expression by *Actinobacillus actinomycetemcomitans* LuxS. *Infect Immun.* 2001; 69:7625–7634. [PubMed: 11705942]
29. Balashova NV, Diaz R, Balashov SV, Crosby JA, Kachlany SC. Regulation of *Aggregatibacter (Actinobacillus) actinomycetemcomitans* leukotoxin secretion by iron. *J Bacteriol.* 2006; 188:8658–8661. [PubMed: 17041062]

30. Isaza MP, Duncan MS, Kaplan JB, Kachlany SC. Screen for leukotoxin mutants in *Aggregatibacter actinomycetemcomitans*: genes of the phosphotransferase system are required for leukotoxin biosynthesis. *Infect Immun*. 2008; 76:3561–3568. [PubMed: 18541661]
31. Mizoguchi K, Ohta H, Miyagi A, Kurihara H, Takashiba S, Kato K, et al. The regulatory effect of fermentable sugar levels on the production of leukotoxin by *Actinobacillus actinomycetemcomitans*. *FEMS Microbiol Lett*. 1997; 146:161–166. [PubMed: 8997720]
32. Inoue T, Tanimoto I, Tada T, Ohashi T, Fukui K, Ohta H. Fermentable-sugar-level-dependent regulation of leukotoxin synthesis in a variably toxic strain of *Actinobacillus actinomycetemcomitans*. *Microbiology*. 2001; 147:2749–2756. [PubMed: 11577154]
33. Kolb A, Busby S, Buc H, Garges S, Adhya S. Transcriptional regulation by cAMP and its receptor protein. *Annu Rev Biochem*. 1993; 62:749–795. [PubMed: 8394684]
34. Gosset G, Zhang Z, Nayyar S, Cuevas WA, Saier MH Jr. Transcriptome analysis of Crp-dependent catabolite control of gene expression in *Escherichia coli*. *J Bacteriol*. 2004; 186:3516–3524. [PubMed: 15150239]
35. Zheng D, Constantinidou C, Hobman JL, Minchin SD. Identification of the CRP regulon using *in vitro* and *in vivo* transcriptional profiling. *Nucleic Acids Res*. 2004; 32:5874–5893. [PubMed: 15520470]
36. Hollands K, Busby SJ, Lloyd GS. New targets for the cyclic AMP receptor protein in the *Escherichia coli* K-12 genome. *FEMS Microbiol Lett*. 2007; 274:89–94. [PubMed: 17608696]
37. Liang W, Pascual-Montano A, Silva AJ, Benitez JA. The cyclic AMP receptor protein modulates quorum sensing, motility and multiple genes that affect intestinal colonization in *Vibrio cholerae*. *Microbiology*. 2007; 153:2964–2975. [PubMed: 17768239]
38. Zhan L, Han Y, Yang L, Geng J, Li Y, Gao H, et al. The cyclic AMP receptor protein, CRP, is required for both virulence and expression of the minimal CRP regulon in *Yersinia pestis* biovar microtus. *Infect Immun*. 2008; 76:5028–5037. [PubMed: 18710863]
39. Spitznagel J Jr, Kraig E, Kolodrubetz D. Regulation of leukotoxin in leukotoxic and nonleukotoxic strains of *Actinobacillus actinomycetemcomitans*. *Infect Immun*. 1991; 59:1394–1401. [PubMed: 2004819]
40. Görke B, Stülke J. Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. *Nat Rev Microbiol*. 2008; 6:613–624. [PubMed: 18628769]
41. Gama-Castro S, Jiménez-Jacinto V, Peralta-Gil M, Santos-Zavaleta A, Peñaloza-Spinola MI, Contreras-Moreira B, et al. RegulonDB (version 6.0): gene regulation model of *Escherichia coli* K-12 beyond transcription, active (experimental) annotated promoters and Textpresso navigation. *Nucleic Acids Res*. 2008; 36:D120–D124. [PubMed: 18158297]
42. Saddi-Ortega L, Carvalho MA, Cisalpino PS, Moreira ES. *Actinobacillus actinomycetemcomitans* genetic heterogeneity: amplification of JP2-like ltx promoter pattern correlated with specific arbitrarily primed polymerase chain reaction (AP-PCR) genotypes from human but not marmoset Brazilian isolates. *Can J Microbiol*. 2002; 48:602–610. [PubMed: 12224559]
43. Chen C, Kittichotirat W, Si Y, Bumgarner R. Genome sequence of *Aggregatibacter actinomycetemcomitans* serotype c strain D11S-1. *J Bacteriol*. 2009; 191:7378–7379. [PubMed: 19820097]
44. Jansen C, Gronenborn AM, Clore GM. The binding of the cyclic AMP receptor protein to synthetic DNA sites containing permutations in the consensus sequence TGTGA. *Biochem J*. 1987; 246:227–232. [PubMed: 3675557]
45. Deutscher J, Francke C, Postma PW. How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. *Microbiol Mol Biol Rev*. 2006; 70:939–1031. [PubMed: 17158705]
46. Seidman CE, Struhl K, Sheen J, Jessen T. Introduction of plasmid DNA into cells. *Curr Protoc Mol Biol*. 1997; 37:1.8.1–2.
47. LeBlanc DJ, Lee LN, Inamine JM. Cloning and nucleotide base sequence analysis of a spectinomycin adenylyltransferase AAD(9) determinant from *Enterococcus faecalis*. *Antimicrob Agents Chemother*. 1991; 35:1804–1810. [PubMed: 1659306]
48. Reytrat JM, Pelicic V, Gicquel B, Rappuoli R. Counterselectable markers: untapped tools for bacterial genetics and pathogenesis. *Infect Immun*. 1998; 66:4011–4017. [PubMed: 9712740]

49. Ferrari, E.; Hoch, JA. Genetics. In: Harwood, CR., editor. *Bacillus*. New York, NY: Plenum Press; 1989. p. 57-72.
50. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics*. 2003; 4:249–264. [PubMed: 12925520]
51. Bolstad BM, Irizarry RA, Åstrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics*. 2003; 19:185–193. [PubMed: 12538238]
52. Saeed AI, Bhagabati NK, Braisted JC, Liang W, Sharov V, Howe EA, et al. TM4 microarray software suite. *Methods Enzymol*. 2006; 411:134–193. [PubMed: 16939790]

Fig. 1.

A *crp* mutant makes less leukotoxin protein. Samples were taken at log phase from anaerobically grown cultures, which contained excess FeCl_3 , of the indicated strains. $\Delta\text{crp}/\text{crp}^+$ is the deletion strain with a crp^+ plasmid in it. Cells were harvested by centrifugation, resuspended in SDS-PAGE loading buffer, boiled, centrifuged and the soluble material was electrophoresed on duplicate 7.5% SDS-polyacrylamide gels. (A) Gel stained with Coomassie Brilliant Blue. (B) Western blot analysis of total cell protein from the indicated strains. The primary antibody was rabbit anti-leukotoxin antibody. The arrow marks the position of leukotoxin (Ltx). The numbers show the positions of molecular size markers (not shown) in kD.

Fig. 2.

Leukotoxin RNA levels are lower in the *crp* deletion mutant. For each sample, total cell RNA was prepared from log phase cells, cDNA was prepared with random hexamer primers and then used in real time PCR. The level of *ltxA* mRNA in each sample is normalized to the level of *pdxY* mRNA in the same sample. The data are the average of the expression ratios from five biological replicates grown as pairs. The error bars are standard deviation. As a control for RNA purity, a parallel set of samples were run without adding reverse transcriptase (RT); in each case the no RT threshold cycle appeared at least eleven cycles later than in the C_t for the corresponding sample with RT (data not shown).

Fig. 3. Proportion of CRP-regulated *A. actinomycetemcomitans* genes in different functional classes. The 2,363 ORFs in *A. actinomycetemcomitans* have been assigned to seventeen broadly defined functional categories (Oral Pathogen Sequence Databases, Los Alamos National Laboratory). Some ORFs have been assigned to two or three functional categories. The “percent in category” for “all genes” is simply the number of *A. actinomycetemcomitans* genes in a particular category as a percentage of the number of ORFs (2,504) in all categories. Similarly, the “percent in category” of CRP-activated and CRP-repressed genes is the number of CRP-activated (or repressed) genes in a particular functional category as a percentage of the total number of ORFs that are CRP-activated (152) or CRP-repressed (169). The total number of CRP-regulated genes is >300 in this calculation because some ORFs have been assigned to more than one functional class.

A. actinomycetemcomitans genes which have potential CRP-binding sites in intergenic regions immediately upstream of CRP-activated operons

Table 1

Operon Number ^d	Gene ID	Gene Information	Fold Change ^b	Position of CRP Site ^c	Patser Score
3	AA00024	Transcriptional regulatory protein	4.80	-199	5.19
	AA00025	Hypothetical protein	4.74		
4	AA00026	Conserved hypothetical protein	32.87	-57	6.11
	AA00028	Conserved hypothetical protein (possible type III restriction- modification system restriction subunit)	31.14		
	AA00029	Type III restriction-modification system: methylase	52.52		
	AA00030	Hypothetical protein	11.87		
5	AA00031	Conserved hypothetical protein	6.32	-92	7.90
	AA00033	Conserved hypothetical protein	17.26		
	AA00034	NTP-binding protein	51.58		
	AA00036	Transposase	14.55		
	AA00037	Conserved hypothetical protein	71.89		
13	AA00497	Hexose phosphate transport system regulatory protein; regulator of <i>uhpT</i> expression	2.61	-81	9.79
14	AA00592	Conserved hypothetical protein (membrane/transport protein)	2.68	-67	10.70
15	AA00632	Inner membrane protein ImpA	19.10	-320	5.29
17	AA00672	Conserved hypothetical protein (possible ppGpp-regulated growth inhibitor)	109.09	-569	5.74
	AA00673	Hypothetical protein	41.13		
18	AA00696	Iron(III) ABC transporter, periplasmic iron-compound-binding protein	5.91	-115	10.65
	AA00698	Iron(III) ABC transporter, periplasmic iron-compound-binding protein	5.41		
	AA00699	Iron(III) ABC transporter,permease protein	3.11		
	AA00700	Cytochrome c-type biogenesis ATP-binding protein	3.12		
19	AA00750	Periplasmic D-galactose-binding ABC transport protein	2.89	-155	10.88
	AA00751	Cytochrome c-type biogenesis ATP-binding protein	2.78		

Operon Number ^a	Gene ID	Gene Information	Fold Change ^b	Position of CRP Site ^c	Patser Score
22	AA00782	Conserved hypothetical protein (possible membrane protein)	77.19	-486	6.24
	AA00785	Conserved hypothetical protein (possible integrase)	329.77		
23	AA00867	Fimbrial protein Flp precursor	2.39	-143	7.07
	AA00868	Possible fimbrial subunit	2.37		
25	AA00978	Conserved hypothetical protein	12.32	-278	5.12
	AA00979	Type B carboxylesterase; p-nitrobenzyl esterase	19.08		
	AA00980	Hypothetical protein	34.63		
	AA00981	Carboxylesterase	32.21		
	AA00982	Aldo-keto reductase	33.78		
	AA00983	Aldo-keto reductase	39.01		
29	AA01318	Hypothetical protein	80.42	+28	6.06
31	AA01378	Glycerol-3-phosphatase transporter	9.09	-108	11.94
	AA01379	Glycerophosphoryl diester phosphodiesterase (protein D)	5.38		
32	AA01383	DNA processing chain A	2.24	-83	11.67
34	AA01549	Hypothetical protein	13.65	-464	6.27
	AA01550	Hypothetical protein	7.10		
37	AA01692	Hexulose-6-phosphate synthase	4.18	-120	12.27
	AA01694	PTS system, IIA component	5.05		
	AA01695	Carbohydrate transport protein	6.52		
39	AA01765	Mu-like prophage FlmMu transposase A	5.26	-337	7.56
41	AA01845	Gamma-glutamyltranspeptidase precursor	2.47	-100	14.79
42	AA01894	Conserved hypothetical protein (possible transthyretin-like periplasmic protein)	4.34	-83	14.45
43	AA01895	Hypothetical protein	2.48	-690	14.45
	AA01896	Hypothetical protein	5.02		
46	AA01902	Conserved hypothetical protein	40.60	-1626	14.42

Operon Number ^a	Gene ID	Gene Information	Fold Change ^b	Position of CRP Site ^c	Pfater Score
47	AA01903	Hypothetical protein	82.03	-1027	14.42
55	AA02149	Possible transposase	35.83	-120	7.80
56	AA02150	Conserved hypothetical protein	116.05	-9	7.80
59	AA02215	Conserved hypothetical protein	4.00	-88	10.82
	AA02217	Deoxyribose-phosphate aldolase	2.97		
	AA02218	Deoxyribose-phosphate aldolase (fragment?)	5.69		
	AA02219	LACI-type transcriptional regulator	3.02		
	AA02220	ABC transport system permease protein	4.09		
	AA02222	Conserved hypothetical protein	3.16		
	AA02224	ABC transport system permease protein	3.61		
	AA02225	ABC transporter ATP-binding protein	6.43		
	AA02226	ABC transporter ATP-binding protein	2.20		
60	AA02232	NagC-like transcriptional regulator	2.98	-96	10.05
61	AA02246	Peptidase E	2.49	-83	9.83
62	AA02249	Thiamine biosynthesis protein	2.25	-133	7.25
64	AA02286	Sugar ABC transporter, ATP-binding protein	14.59	-273	12.14
67	AA02473	Conserved hypothetical protein	2.22	-122	10.64
	AA02476	Conserved hypothetical protein	2.63		

^aThese operon numbers correspond to the operon numbers assigned in Table S1. They are not consecutive numbers in this table because a lot of operons do not have a close-by CRP-binding site and so are not included in this table.

^bRatio (JP2/ Δcrp).

^cThe position of the CRP site is relative to the ATG of the first gene in the operon.

A. actinomycetemcomitans genes which have potential CRP-binding sites in intergenic regions immediately upstream of CRP-repressed operons

Table 2

Operon Number ^d	Gene ID	Gene Information	Fold Change ^b	Position of CRP Site ^c	Patser Score
86	AA00069	Conserved hypothetical protein	2.02	-149	10.95
92	AA00207	Cytochrome c-type biogenesis ATP-binding protein	32.82	-87	10.81
	AA00208	D-ribose ABC transporter, permease protein / Galactoside transport system permease protein	37.42		
	AA00209	D-ribose ABC transporter, periplasmic-binding protein	81.55		
	AA00211	Hypothetical protein	110.58		
	AA00212	Ribokinase	45.66		
94	AA00236	Anaerobic ribonucleoside-triphosphate reductase	2.08	+33	6.42
98	AA00429	Conserved hypothetical protein	5.76	-459	14.60
100	AA00452	Phosphomannomutase; phosphoglucomutase	2.36	-104	9.41
104	AA00634	DNA polymerase IV (Pol IV)	13.15	-38	5.13
105	AA00663	Cystathionine beta-lyase	2.36	-33	7.00
109	AA00781	Hypothetical protein	8.92	-106	6.19
120	AA01135	Possible cell filamentation protein	3.92	-131	5.47
121	AA01137	Anaerobic dimethyl sulfoxide reductase, chain A	2.18	-80	11.44
	AA01138	Anaerobic dimethyl sulfoxide reductase, chain B	2.33		
	AA01141	Conserved hypothetical protein (possible component of anaerobic dehydrogenase/oxidoreductase)	2.43		
129	AA01455	D-xylose ABC transporter, ATP-binding protein	3.18	-150	10.70
	AA01456	D-xylose ABC transporter, ATP-binding protein	2.35		
130	AA01465	Mannose-specific phosphotransferase element	2.05	-100	14.46
	AA01466	Mannose-specific phosphotransferase system IIC	2.08		
	AA01467	Mannose-specific phosphotransferase system IID	2.11		

Operon Number ^a	Gene ID	Gene Information	Fold Change ^b	Position of CRP Site ^c	Patser Score
131	AA01544	Conserved hypothetical protein	2.50	-65	9.71
134	AA01696	Conserved hypothetical protein	2.88	-226	12.27
	AA01698	Probable transcriptional regulator (DeoR family)	2.10		
136	AA01717	Fumarate hydratase, class II	2.21	-100	10.16
141	AA01875	PTS system, glucose-specific enzyme II, A component	2.08	-121	9.26
	AA01876	PTS system, IIBC components	2.52		
143	AA01918	Anaerobic C4-dicarboxylate transporter	2.21	-94	9.72
144	A01923	Type III restriction-modification system methylation subunit	6.37	-78	5.76
	AA01928	ATP-dependent RNA helicase	4.78		
145	AA01938	NAD(P) transhydrogenase subunit alpha	2.01	-142	8.07
146	AA01944	Conserved hypothetical protein	2.10	-186	5.53
153	AA02193	Oxaloacetate decarboxylase gamma chain	2.39	-69	10.15
	AA02195	Oxaloacetate decarboxylase alpha chain	2.30		
	AA02196	Oxaloacetate decarboxylase beta chain	3.33		
154	AA02201	Transferrin-binding protein I precursor	21.99	-44	5.32
	AA02202	Transferrin-binding protein I precursor	41.76		
156	AA02210	Ribonucleoside-diphosphate reductase alpha chain	3.35	-61	10.09
	AA02212	Ribonucleoside-diphosphate reductase beta chain	4.59		
157	AA02237	Molybdopterin biosynthesis protein E chain	4.15	-198	7.38
	AA02239	Molybdopterin biosynthesis protein D	3.80		
	AA02241	Molybdenum cofactor biosynthesis protein C	4.46		
	AA02242	Molybdenum cofactor biosynthesis protein A	3.90		
158	AA02250	Possible cytochrome c-type protein	13.14	-101	6.40
	AA02251	Biotin sulfoxide reductase; rimethylamine-N-oxide reductase precursor	15.92		

Operon Number ^a	Gene ID	Gene Information	Fold Change ^b	Position of CRP Site ^c	Patser Score
162	AA02393	Conserved hypothetical protein; possible sulfate transporter	2.42	-115	5.11
166	AA02577	Nuclease	4.82	-297	8.88
	AA02579	Aminotransferase nifS protein	3.68		
168	AA02671	Glucononase	2.11	-131	11.29
169	AA02683	Hydrogenase 4 Fe-S subunit	3.08	-101	8.01
	AA02687	Hydrogenase-4 component D	3.29		
	AA02688	Hydrogenase 4 component E	4.02		
	AA02689	Hydrogenase-4 component F	3.46		
	AA02692	Hydrogenase 4 subunit; hydrogenase 3 subunit	4.70		
	AA02693	Hydrogenase-4 component H	2.81		
	AA02696	Formate hydrogenlyase maturation protein	3.92		
	AA02697	Hydrogenase 3 maturation protease	4.26		
171	AA02726	Anaerobic C4-dicarboxylate transporter	2.34	-100	5.48
172	AA02756	Phosphoserine aminotransferase	5.47	-140	9.51
	AA02757	Histidinol-phosphate aminotransferase 2	5.22		
	AA02758	3-phosphohikimate 1-carboxyvinyltransferase	2.68		
173	AA02768	Conserved hypothetical protein	2.66	-399	10.14
174	AA02772	Hypothetical protein	2.53	-119	10.74
175	AA02784	NAD ⁺ dependent acetaldehyde-alcohol dehydrogenase, iron-containing	3.59	-70	10.46

^aThese operon numbers correspond to the operon numbers assigned in Table S1. They are not consecutive numbers in this table because a lot of operons do not have a close-by CRP-binding site and so are not included in this table.

^bRatio ($\Delta crp/JP2$).

^cThe position of the CRP site is relative to the ATG of the first gene in the operon.