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Published in: Journal of Bacteriology

DOI: 10.1128/JB.01049-06

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Document Version
Publisher’s PDF, also known as Version of record

Publication date: 2006

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Iron Starvation Triggers the Stringent Response and Induces Amino Acid Biosynthesis for Bacillibactin Production in Bacillus subtilis

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Received 18 July 2006/Accepted 20 September 2006

Iron deprivation in bacteria causes the derepression of genes controlled by the ferric uptake regulator (Fur). The present microarray analysis of iron-starved Bacillus subtilis cells grown in minimal medium unveils additional physiological effects on a large number of genes linked to stringent-response regulation and to genes involved in amino acid biosynthesis associated with pathways essential for bacillibactin production.

Iron is an essential cofactor in various biosynthetic and bioenergetic pathways. In many bacteria, the Fur-dependent derepression of iron acquisition genes is a common strategy used to overcome iron starvation (13). The Fur regulon of B. subtilis comprises 39 genes coding mainly for siderophore biosynthesis and several iron transporters (1). The Escherichia coli Fur protein controls an even larger regulon of similar constitution and was shown to regulate the cellular iron-protein content (17). This study investigated the consequences of permanent iron depletion under nonrich growth conditions on global gene expression and shows a complex physiological response beyond Fur regulation.

For our analyses, Bacillus subtilis strain ATCC 21332 (sfp+) (7) was grown in a defined medium without (iron-depleted cultures) or with (iron-replete cultures) the addition of 10 μM FeSO₄ as described previously (18). The growth rates of iron-depleted and iron-replete cultures were similar until the late exponential phase. The growth of the iron-depleted culture then declined and, in contrast to that of the iron-replete culture, catechol siderophore secretion greatly increased (see Fig. S1 in the supplemental material), confirming iron as the limiting nutrient in the late exponential phase. At this time point (optical density at 600 nm, ~0.35), the mRNA populations of iron-depleted and iron-replete cultures were compared by microarray analysis (essentially performed as described previously [15; see also technical details in the supplemental material]). Genes of several prominent functional and regulatory classes exhibited iron-dependent repression or induction (see Tables S1 and S2 in the supplemental material). As expected, iron starvation led to induction of the Fur regulon, although there were slight differences from previous studies of Fur regulation carried out with B. subtilis 168 (sfp) in broth medium (1). Interestingly, among the Fur-dependent ABC transporters, the feuABC genes for ferri-bacillibactin uptake (18, 20) showed the strongest induction. In contrast, the fhuBGC-fhuD genes for ferrichrome uptake (24) were only slightly induced and the yfmCDEF and yflYZ-yflA ferric iron transporter genes were not induced. This might indicate a hierarchical expression of iron transporters when dominant iron chelators such as bacillibactin are present. Additionally, 24 genes were upregulated that were reported to be regulated by the transcriptional repressor CodY (19). It has been suggested that derepression of GTP-activated CodY is mediated by a decreasing cellular GTP pool upon RelA-dependent formation of (p)ppGpp, the second messenger of the stringent response (21). To investigate this relationship more closely, we compared our transcriptome data with a global stringent-response analysis (8). Indeed, there was a high coincidence of gene regulation between the two studies. Among the genes earlier described to be either positively or negatively RelA regulated, we found 19 and 28 genes to be up- and downregulated during iron starvation, respectively. Furthermore, among the genes that were reported to be affected independently of RelA during the stringent response, we found 26 genes showing a similar iron-dependent repression or induction. These genes mainly belonged to the functional categories of amino acid, purine, and pyrimidine biosynthesis. To confirm these and further results of the microarray analysis, iron-dependent repression or induction of selected genes was subsequently compared by an independent dot blot analysis (Fig. 1; see technical details in the supplemental material). The specific transcript detection showed the same expression pattern for both the RelA-dependent genes rpsP and ald and the CodY-dependent genes ipdV and yurO, as revealed by the transcriptome analysis. Several enzymes in amino acid biosynthesis pathways are iron dependent, and iron limitation may subsequently cause amino acid starvation. The most abundant amino acid in both gram-positive and gram-negative bacteria is glutamate. Especially Bacillus spp. need a large intracellular glutamate pool (40 to >100 mM) for vegetative growth and adaptational processes (25). Since B. subtilis lacks an anabolic glutamate dehydrogenase (3), glutamate synthesis is strictly iron dependent as iron is needed to assemble the iron-sulfur cluster bound to glutamate synthase as a cofactor (26). The gltA and gltB genes coding for the large and small chains of the B. subtilis glutamate synthase, respectively, were downregulated during iron starvation.

† Supplemental material for this article may be found at http://jb.asm.org/.

‡ Published ahead of print on 29 September 2006.
depletion (see also Fig. 1). The expression of the *gltAB* operon, which is triple regulated by TnrA (4), GltC (6), and CcpA (9), depends on a sufficient supply of ammonium and glucose (5, 28). Since both ammonium and glucose were present at non-limiting concentrations in the minimal medium used, the underexpression of *gltAB* seems to be a direct result of low iron availability. Furthermore, we found that the *citB* gene coding for the iron-dependent *B. subtilis* aconitase involved in substrate supply for GltA was also downregulated by iron depletion, as observed in previous work (1). *citB* repression seems to be directly iron dependent, since further genes of the tricarboxylic acid cycle that are essential for the synthesis of the bacillibactin precursors threonine and glycine, as shown schematically in Fig. 2. Threonine synthesis starting from aspartate needs five enzymatic activities. Five genes coding for four of these activities were upregulated: *lysC* (aspartokinase II) and *yclM* (aspartokinas e III, *thrD*), encoding two isozymes for the initial reaction (2, 10), as well as *hom*, *thrB*, and *thrC*, coding for homoserine dehydrogenase, homoserine kinase, and threonine synthase, respectively. In the synthesis pathway leading from 3-phosphoglycerate via serine to glycine, the *yoaD* gene, coding for a putative paralog of the initial enzyme SerA, was upregulated. The genes *yclM, hom*, and *yoaD* were selected for dot blot analysis (Fig. 1). In the amino acid biosynthesis network, the threonine, serine/glycine, and cysteine/methionine pathways are interdependent. In total, there are seven specific enzymatic activities needed for cysteine/methionine synthesis. The genes *ycl*, *yjcJ*, *yitJ* (12), and *cysE*, coding for four of these activities, were upregulated. Additionally, *yxjG*, coding for a protein similar to the methionine synthase MetE, possibly provides a further activity to this pathway. Altogether, 9 out of the 11 genes are either S box (*yjcJ, yjcI, yitJ, yoaD*, and *yxjG*) or T box (*hom*,

**FIG. 1.** Dot blot analysis of selected genes. Shown are the relative transcriptional levels of cells cultured in minimal medium under iron-starved (−) or iron-replete (+) conditions. Panels: A, Fur-regulated genes (as controls); B, amino acid biosynthesis genes of the threonine (*yclM, hom, yoaD, gltA*) pathways; C, stringent-response-regulated genes; D, CodY-regulated genes; E, genes of the tricarboxylic acid cycle. The arrows indicate increased (↑), decreased (↓), or equal (→) transcript amounts that were detected during iron starvation in comparison with iron-replete conditions.
thiR, thiC, and cysE) regulated (11, 12). Furthermore, yjcJ, yjcI, yitJ, and youD were shown to be induced RelA independently during the stringent response (8). Thus, in addition to these regulatory mechanisms, it is tempting to speculate that there could be a specific link between iron starvation and/or bacilli-bactin synthesis and the threonine, serine/glycine, and cysteine/methionine pathways. However, because of the moderate induction of the precursor biosynthesis genes, a more obvious explanation might be the occurrence of S- and T-box-dependent feedback regulation(s) caused by the consumption of threonine, glycine, and serine (as glycine precursor) in bacilli-bactin synthesis, thus leaving a “regulatory footprint” in the primary metabolism. However, this is the first time that siderophore synthesis-dependent regulation in the primary metabolism was observed, underlining the importance of bacilli-bactin as a major iron deficiency rescue system in \textit{B. subtilis}.

In conclusion, the results of this study show the relevance of both culture medium composition and the capability of siderophore production to global gene expression during iron starvation and establish novel iron-dependent functional and regulatory connections between differentially classified genes.

We thank the group of E. Bremer (Marburg) for help with RNA preparation and chemiluminescence detection. Anne de Jong, Siger Holappa, and Anne Sadewasser are acknowledged for technical assistance.

This work was supported by EC grant LSHG-CT-2004-503468.

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