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Iron Starvation Triggers the Stringent Response and Induces Amino Acid Biosynthesis for Bacillibactin Production in *Bacillus subtilis* \(^7\)†

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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, \(\sim 0.35\)), 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\(^\theta\)) 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 _yfYZ-yfhA_ 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 _glaA_ and _glhB_ genes coding for the large and small chains of the *B. subtilis* glutamate synthase, respectively, were downregulated during iron...
Altogether, carbon and nitrogen sources as \textit{citB} for the iron-dependent biont, as observed in previous work (1). The genes \textit{gltAB} were not affected, such as \textit{citB} availability. Furthermore, we found that the \textit{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 \textit{gltAB} seems to be a direct result of low iron availability. Furthermore, we found that the \textit{citB} gene coding for the iron-dependent \textit{B. subtilis} aconitase involved in substrate supply for \textit{GltAB} was also downregulated by iron depletion, as observed in previous work (1). \textit{citB} repression seems to be directly iron dependent, since further genes of the tricarboxylic acid cycle were not affected, such as \textit{citZ}, which is regulated by the same carbon and nitrogen sources as \textit{citB} (see also Fig. 1) (14, 22). Altogether, \textit{gltAB} and \textit{citB} downregulation indicates an iron-dependent bottleneck of glutamate synthesis. A recent study demonstrating SpoT-dependent (p)ppGpp accumulation during iron limitation in \textit{E. coli} (27) might indicate similar effects in gram-negative bacteria. However, the stringent response observed in \textit{B. subtilis} during iron starvation is most likely the consequence of deficiencies in iron-dependent amino acid biosynthesis.

Recently, ferri-bacillibactin was shown to be the major endogenously derived iron source of \textit{B. subtilis} during iron starvation (18). The utilization of this nonribosomally produced catecholic trilactone (2,3-dihydroxybenzoate-glycine-threonine), siderophore is mediated by the Fur-regulated \textit{FeuABC} uptake system and the BesA trilactone hydrolase (18, 20). While the \textit{dhbACEBF} operon coding for 2,3-dihydroxybenzoate synthesis (23) and bacillibactin assembly (16) is also controlled by Fur (1), iron-dependent regulation of primary metabolic genes involved in bacillibactin synthesis was not reported. In total, our transcriptome study revealed 11 amino acid biosynthesis genes that were more than 40% upregulated during iron starvation. Strikingly, all of them code for enzymes 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: \textit{lysC} (aspartokinase II) and \textit{yclM} (aspartokinase III, \textit{thrD}), encoding two isozymes for the initial reaction (2, 10), as well as \textit{hom}, \textit{thrB}, and \textit{thrC}, coding for homoserine dehydrogenase, homoserine kinase, and threonine synthase, respectively. In the synthesis pathway leading from 3-phosphoglycerate via serine to glycine, the \textit{yodD} gene, coding for a putative paralog of the initial enzyme \textit{SerA}, was upregulated. The genes \textit{yclM}, \textit{hom}, and \textit{yodD} 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 \textit{yjcl}, \textit{yjcl}, \textit{yjcl} (12), and \textit{cysE}, coding for four of these activities, were upregulated. Additionally, \textit{yjG}, 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 \textit{S} box (\textit{yjcl}, \textit{yjcl}, \textit{yjcl}, \textit{yodD}, and \textit{yjG}) or \textit{T} box (\textit{hom},

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**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 (\textit{yclM, hom, yoaD}), serine/glycine (\textit{yodD}), and glutamate (\textit{glta}) pathways; C, stringent-response-regulated genes; D, CodY-regulated genes; E, E. coli-dependent bottleneck of glutamate synthesis. The arrows indicate increased (↑), decreased (↓), or equal (→) transcript amounts that were detected during iron starvation in comparison with iron-replete conditions.

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**FIG. 2.** Scheme of bacillibactin synthesis. The bacillibactin precursors glycine and threonine derive from the primary metabolism. The synthesis of 2,3-dihydroxybenzoate (DHB) and the nonribosomally catalyzed bacillibactin assembly is also controlled by Fur (1), iron-dependent regulation of primary metabolic genes involved in bacillibactin synthesis was not reported. In total, our transcriptome study revealed 11 amino acid biosynthesis genes that were more than 40% upregulated during iron starvation. Strikingly, all of them code for enzymes 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: \textit{lysC} (aspartokinase II) and \textit{yclM} (aspartokinase III, \textit{thrD}), encoding two isozymes for the initial reaction (2, 10), as well as \textit{hom}, \textit{thrB}, and \textit{thrC}, coding for homoserine dehydrogenase, homoserine kinase, and threonine synthase, respectively. In the synthesis pathway leading from 3-phosphoglycerate via serine to glycine, the \textit{yodD} gene, coding for a putative paralog of the initial enzyme \textit{SerA}, was upregulated. The genes \textit{yclM}, \textit{hom}, and \textit{yodD} 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 \textit{yjcl}, \textit{yjcl}, \textit{yjcl} (12), and \textit{cysE}, coding for four of these activities, were upregulated. Additionally, \textit{yjG}, 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 \textit{S} box (\textit{yjcl}, \textit{yjcl}, \textit{yjcl}, \textit{yodD}, and \textit{yjG}) or \textit{T} box (\textit{hom},
starvation and establish novel iron-dependent functional and siderophore production to global gene expression during iron metabolism was observed, underlining the importance of bacillibactin 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 bacillibactin 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 bacillibactin as a major iron deficiency rescue system in 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.

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