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Bacillibactin-Mediated Iron Transport in *Bacillus subtilis*[1]

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Iron, although the fourth most abundant metal in the earth's crust, is usually a growth-limiting nutrient for microorganisms, including many human pathogens.[2,3] First characterized as iron transport agents in the 1960s, siderophores are low molecular weight chelators that are very selective for Fe^{3+}.[4,5] Enterobactin (Ent, Figure 1), produced primarily by Gram-negative bacteria, is perhaps the best understood siderophore and was thought to be unique in its iron affinity.[6] However, a siderophore remarkably similar to Ent was reported as isolated from *Corynebacterium glutamicum* and termed "corynebactin".[7] It was later reported that *Bacillus subtilis* produces this same iron chelator, with the name bacillibactin proposed (BB, Figure 1).[8] On the basis of genome sequence and siderophore uptake assays, we have now determined that *C. glutamicum* apparently does not produce this siderophore, and so we adopt the use of the name bacillibactin.[9] Like Ent, BB incorporates a trilactone ring and three catecholate moieties. However, the structure of BB exhibits two striking differences: the trilactone ring is methylated, and the arms contain a glycine spacer between the catecholamide and the ring. The predisposition of Ent for Fe^{3+} results in a phenomenally high complex stability; so what is the effect of alteration of this apparently optimum structure by elongation of the arms and methylation of the ring?

Although many aspects of *B. subtilis* biochemistry, physiology, and genetics have been intensely studied, its iron-acquisition systems have not been well described.[10,11] Until 2000, when May et al. reported the biosynthetic pathway of BB,[8] only one endogenous siderophore (itoic acid (IA), Figure 1) had been characterized in *B. subtilis*.[12] While many publications describe siderophore transport in Gram-negative microorganisms, much less is known for Gram-positive bacteria, such as *B. subtilis*. Iron uptake experiments with *B. subtilis* using BB, Ent, IA, and enantioenterobactin (Enantio) indicate that this organism can recognize a variety of catecholate siderophores, but does so through the expression of several and sometimes overlapping membrane transport proteins.

Ligand protonation constants were determined for BB, and the ferric formation and protonation constants were evaluated. Three of the six protonation constants for BB have been determined potentiometrically (Table 1).[13] Previous work with Ent and its analogues determined that the protonation constants (pK_{a1}−pK_{a3}) of the *m*-hydroxy oxygen atoms are well separated from the *o*-hydroxy oxygen atoms (pK_{a4}−pK_{a6}). Thus, stepwise protonation constants (for $n = 1-6$) of the ligand are available for the reactions

$$H^+ + H_{n-1}L^{n-7} = H_nL^{n-6} \qquad (1)$$

The FeBB stability constant was measured via a competition experiment with EDTA.[14] By monitoring the visible spectrum, the amount of ferric siderophore complex formed was determined:

$$FeEDTA^- + H_6L = FeL^{3-} + H_2EDTA + 4H^+ \quad K_{comp} \qquad (2)$$

A solution of Fe, BB, and EDTA (0.9:1:20) was divided into 6 aliquots, and base was added to each sample to give a pH range from 5.2 to 7.5. After a 36 h equilibration time, each spectrum and pH were recorded to ascertain the ferric formation constant for BB (Figure 2, left). The proton-independent stability constant for the FeBB is then calculated (Table 1):[15]

$$Fe^{3+} + L^{6-} = FeL^{3-} \quad K_f = \beta_{110} = 10^{47.6(1)} \qquad (3)$$

A solution of Fe and BB (~1:1) was divided into 6 aliquots, and

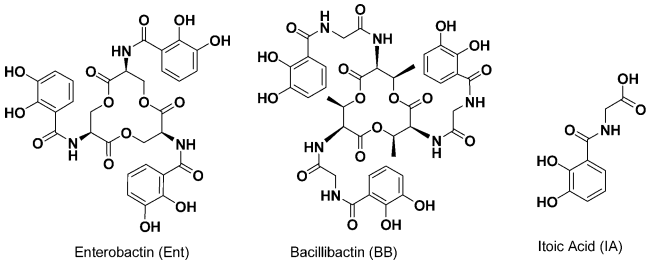

Figure 1. Enterobactin (Ent), Bacillibactin (BB), and Itoic Acid (IA).

Table 1. Solution Thermodynamic Data for BB and Ent[a]

pK_a	BB	Ent[18]
$\log K_1$	12.1	12.1
$\log K_2$	12.1	12.1
$\log K_3$	12.1	12.1
$\log K_4$	8.43 (2)	8.6
$\log K_5$	7.43 (2)	7.5
$\log K_6$	6.77 (3)	6.0
$\Sigma \log K_{1-6}$	58.93	58.4
$\log \beta_{110}$	47.6(1)	49
$\log \beta_{111}[pK_{a1}]$	52.9(1) [5.3]	54 [5.0]
pM	33.1	34.3

[a] The pK_{a1-3} values are estimates based upon bidentate analogues[18] (0.1 M KCl, 25 °C).

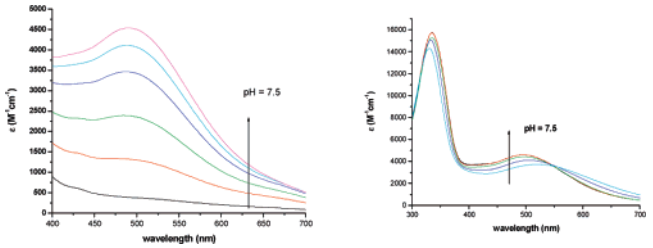

Figure 2. Spectrophotometric titrations (0.1 M KCl, 25 °C, 1 cm cell). (Left) EDTA competition to determine β_{110} for FeBB ([BB] = 0.13 mM, $[Fe^{3+}]$ = 0.11 mM, [EDTA] = 2.6 mM, pH 5.2−7.5, 36 h). (Right) FeBB pK_a ([BB] = 0.12 mM; $[Fe^{3+}]$ = 0.11 mM, pH from 4.2 to 7.5, 36 h).

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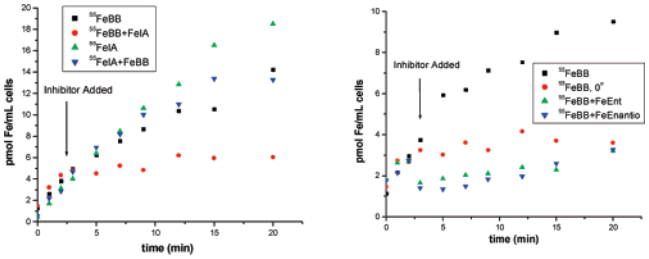

Figure 3. Iron transport mediated by (left) BB and IA and (right) BB (0.9 μM) in *B. subtilis*. The inhibitor ($\times$15 excess of NR FeBB, FeIA, FeEnt, or FeEnantio) was added at 2.5 min. Data presented are the average of two independent experiments.

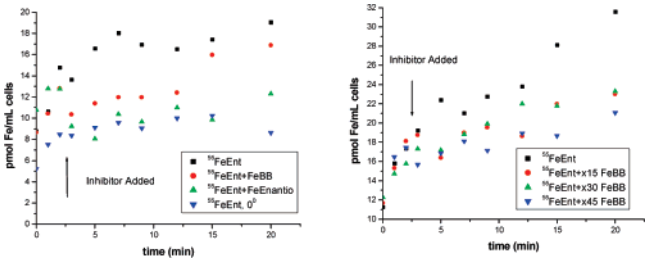

Figure 4. Iron transport mediated by Ent (0.9 μM) in *B. subtilis*. The inhibitor (left, $\times$15 excess NR FeEnantio or FeBB; or right, $\times$15, 30, or 45 excess of NR FeBB) was added at 2.5 min. Data presented are the average of two independent experiments.

base was added to each sample to give a pH range from 4.2 to 7.5. After a 36 h equilibration time, each spectrum and pH were recorded to ascertain the ferric complex protonation constant (Figure 2, right). Deconvolution of the spectral data yielded a pK_a value of 5.3 for FeBB (Table 1).[15]

Although IA and BB are the endogenous siderophores of *B. subtilis*, competitive uptake experiments have not yet been reported. Nonradioactive (NR) FeIA blocks the uptake of ^{55}FeBB, but NR FeBB does not completely block the incorporation of ^{55}FeIA (Figure 3, left). Thus, at least two catecholate permeases are present: one transports IA only (permease 1) and the other transports IA and BB (permease 2).

Chirality at the metal center can be a distinguishing feature recognized by some siderophore receptors. FeBB and FeEnt have opposite chirality[16] and different shapes, with the glycine spacer forcing FeBB to be more oblate as compared to FeEnt.[17] These changes could force the organism to incorporate one siderophore preferentially over the other. Addition of both NR FeEnt and FeEnantio impede the incorporation of ^{55}FeBB, indicating that FeBB shares a common membrane permease with Ent and Enantio. The different chirality of Ent as compared to that of BB does not appear to diminish the competition for the permease since Ent was as effective as Enantio in blocking BB uptake (Figure 3, right).

To further probe the role of chirality, NR FeEnantio was added to a bacterial suspension of *B. subtilis* supplied with ^{55}FeEnt and vice versa. In each case, the NR enantiomer effectively blocked the transport of its mirror image (Figure 4, left, shown for ^{55}FeEnt). The complementary competition experiments with NR FeBB added to block ^{55}FeEnt and ^{55}FeEnantio uptake revealed that FeBB does not completely block the transport of FeEnt and FeEnantio (Figure 4, left, shown for ^{55}FeEnt). There are two possible explanations. First, the permease could have a higher affinity for Ent and its enantiomer than for BB. Second, *B. subtilis* could possess two catecholate transporters (similar to permeases 1 and 2 found for BB and IA), one mediating the uptake of FeBB, FeEnt, and FeEnantio (permease 2) and another transporting just FeEnt and FeEnantio (permease 3).

The first possibility was explored by increasing the concentration of NR FeBB in the uptake bacterial suspension. However, the addition of 15-, 30-, and 45-fold excess of NR FeBB did not change the uptake profile of either ^{55}FeEnt or ^{55}FeEnantio (Figure 4, right, shown for ^{55}FeEnt), supporting the presence of at least two transporters (permeases 2 and 3).

The structural changes between BB and Ent result in these two siderophores having differing shapes and differing affinities for iron and requiring multiple, but partially overlapping pathways for iron incorporation. The trilactone backbone of Ent appears perfect for the size of the ferric ion, and addition of the glycine spacer is detrimental to the overall stability, evident in the lowered thermodynamic stability of FeBB compared to that of FeEnt. Three catecholate transport mechanisms are operative in *B. subtilis*: one for Ent, one for IA, and the third transports IA, Ent, and BB.[19] This behavior is similar to that found for Gram-negative *Salmonella typhimurium*, which use both FeEnt and FeBB by expressing two receptor proteins: IroN and FepA.[20] While Ent is transported by both receptors, only IroN transports BB, with the difference in shape key to the recognition.

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Supporting Information Available: Detailed experimental procedures for titration data and siderophore uptake studies. Additional uptake data for enantioenterobactin are also available. This material is available free of charge via the Internet at http://pubs.acs.org.

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