J Biol Inorg Chem (2006) 11:1087–1097
DOI 10.1007/s00775-006-0151-4

ORIGINAL PAPER

Siderophore-mediated iron transport in *Bacillus subtilis* and *Corynebacterium glutamicum*

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Received: 19 April 2006 / Accepted: 21 July 2006 / Published online: 16 August 2006

Abstract Hexadentate bacillibactin is the siderophore of *Bacillus subtilis* and is structurally similar to the better known enterobactin of Gram-negative bacteria such as *Escherichia coli*. Although both are triscatecholamide trilactones, the structural differences of these two siderophores result in opposite metal chiralities, different affinity for ferric ion, and dissimilar iron transport behaviors. Bacillibactin was first reported as isolated from *Corynebacterium glutamicum* and called corynebactin. However, failure of iron-starved *C. glutamicum* to transport ^{55}Fe bacillibactin and lack of required bacillibactin biosynthetic genes suggest that bacillibactin is not the siderophore produced by this organism. Iron transport mediated by siderophores in *B. subtilis* occurs through a transport process that is specific for the iron chelating moiety, with parallel pathways for catecholates and hydroxamates. For bacillibactin, enterobactin, and their analogs, neither chirality nor presence of an amino acid spacer affects the uptake and transport process, but alteration of the net charge and size of the molecule impedes the recognition.

Keywords Bacillibactin · Corynebactin · Gram-positive · Iron transport · Siderophore

Paper number 77 in the series Coordination Chemistry of Microbial Iron Transport Compounds. See Abergel et al. [1].

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Introduction

Despite the variety of microbial niches, one common challenge for virtually all species is the bioavailability of iron [2]. Although iron is the fourth most abundant metal in the earth's crust, it exists primarily as insoluble hydroxides in aerobic aqueous solution, making its acquisition difficult for microorganisms. To overcome this challenge, many microorganisms secrete low molecular weight iron chelators called siderophores [3]. After sequestering available iron, the ferric complex is incorporated into the cell, and iron is released from the siderophore by reduction of the iron [2] or hydrolysis of the siderophore [4].

In 1997, Budzikiewicz et al. [5] reported isolating the siderophore corynebactin, from *Corynebacterium glutamicum*, a Gram-positive bacterium used in the industrial production of glutamic acid for monosodium glutamate. [Confusingly, this name was also used for the structurally uncharacterized (and apparently unrelated) siderophore from *C. diphtheriae* [6].] May et al. [7] later reported isolation of the same compound from *Bacillus subtilis* and renamed it bacillibactin (BB). On the basis of the study reported here, we recommend using the name ''bacillibactin'' for the catecholamide

trimer produced by *B. subtilis* and ''corynebactin'' for the structurally uncharacterized siderophore of *C. diphtheriae*. That usage will be throughout this paper. As in enterobactin, the structure of BB also incorporates a trilactone ring and three catecholate binding groups (Fig. 1); 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 production of the same siderophore in two unrelated and taxonomically distinct Gram-positive organisms was surprising and prompted this examination of the BB-mediated iron transport pathways in *B. subtilis* and *C. glutamicum* [8]. Although iron transport in *C. glutamicum* has not been previously reported, many researchers have investigated the iron requirements and acquisition methods of *C. diphtheriae*, the causative agent of diphtheria [9]. Iron availability and *C. diphtheriae* virulence have been linked since 1936, when Pappenheimer and Johnson [10] showed that toxin production was maximal at low iron conditions and inhibited in iron-replete medium. Only *C. diphtheriae* infected with the tox^+ corynephage makes and secretes diphtheria toxin [11, 12]. In the presence of excess iron, diphtheria toxin regulator (DtxR) binds Fe^{2+} and represses toxin production by binding to the *tox* operator site [13]. In the absence of iron, DtxR dislocates and the toxin is produced. DtxR is known to regulate siderophore-related genes in *C. diphtheriae* [14] and was recently reported to regulate 26 genes that encode iron transport systems or siderophore interacting proteins (however no mention was made of the siderophore named corynebactin) in *C. glutamicum* [15]. The genes involved in iron acquisition in *B. subtilis* are regulated by ferric uptake regulator (Fur) [16]. Fur and DtxR represent two distinct prototypes for a common biological goal: the response to iron stress.

In 1984, Russell et al. [6] isolated a siderophore from *C. diphtheriae*, and partially characterized the binding groups using the Arnow [17] (vic-diols) and Csaky [18] (hydroxamic acids) tests. Both tests were negative, but later the universal Chrome Azurol S (CAS) [19] siderophore assay, which indicates the ability to remove iron from the CAS chromophore rather than the specific iron chelating moiety [20], was positive and so the compound was characterized as a siderophore and called corynebactin. A seven-gene cluster (*ciuABCDEFG*) was identified that was regulated by DtxR and found to be responsible for the biosynthesis and transport of this siderophore [21].

Bacillus species have received a moderate amount of attention with respect to iron transport mechanism. Itoic acid (Fig. 1), the first example of a catecholate siderophore, was isolated from *B. subtilis* in 1958 [22], and BB was characterized in 2000 [7]. A monomeric unit of BB (2,3-dihydroxybenzoyl glycylthreonine) was isolated from *B. licheniformis* [23]. An active transport process in *B. subtilis* was demonstrated in the early 1970s [24–27] and the subsequent link between environmental iron concentrations and production of phenolic acids was demonstrated [28–30]. Walsh et al. [30] recognized that multiple parallel iron uptake pathways would explain the repression of 2,3-dihydroxybenzoyl synthesis by the hydroxamate siderophore, ferrichrome. As long as the organism had access to iron, siderophore production activity could be reduced.

To provide a more complete picture of siderophore-mediated iron transport in *B. subtilis* and *C. glutamicum*, iron uptake was further explored using BB, other siderophores, and siderophore analogs. In contrast to the initial report, production of BB by *C. glutamicum* could not be replicated and incorporation of ferric BB was not evident. In addition, the analysis of the *C. glutamicum* genome reveals the absence of the genes required for the biosynthesis of BB [31, 32]. *B. subtilis*, however, both produces and utilizes BB and is able to utilize a variety of ligands to accumulate iron.

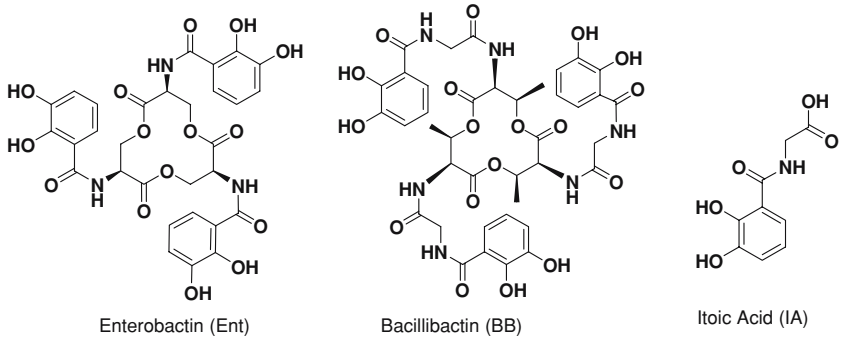

Fig. 1 Siderophores enterobactin (from *Escherichia coli*), bacillibactin (from *Bacillus subtilis*), and itoic acid (from *B. subtilis*)

Materials and methods

General

Unless otherwise noted, starting materials were obtained from commercial suppliers and used without further purification.

Bacterial growth procedures

C. glutamicum ATCC 14607 and *B. subtilis* ATCC 6051 were acquired from the American Type Culture Collection and routinely cultured on Luria–Bertani agar plates at 37 °C. Iron-limited growth medium was prepared according to the following method: glucose (5 g/L), Difco bacto casamino acid (3 g/L), $(NH_4)_2HPO_4$ (1 g/L), K_2HPO_4 (2.5 g/L), KH_2PO_4 (2.5 g/L), nicotinic acid (40 μM), and thiamine (100 μM) were dissolved in ddH_2O and the pH was adjusted to 6.8. This solution was passed through a Chelex100 column to remove any iron and then filter-sterilized. Filter-sterilized $MnSO_4$ (36 μM), $ZnSO_4$ (0.3 μM) and $MgSO_4$ (830 μM) were then added to the medium. Iron-replete medium was prepared as iron-free medium, but also included the addition of $FeCl_3$ to a final concentration of 100 μM.

Inoculation

One 125-mL Erlenmeyer flask containing 50 mL of the low-iron medium was inoculated with *C. glutamicum* ATCC 14607 or *B. subtilis* ATCC 6051 and incubated at 37 °C for 15 h (with shaking at 200 rpm). For siderophore isolation, 25 mL of this preculture was used to inoculate 500 mL of the iron-limited medium, which was incubated at 37 °C for 48 h (with shaking at 200 rpm). For siderophore uptake studies, 1 mL of the preculture was used to inoculate 150 mL of iron-limited medium, and each culture was grown to the late exponential phase (optical density at 600 nm, OD_{600}, of 0.6).

Siderophore isolation from *B. subtilis*

After 48 h of growth, the culture medium (500 mL) was checked for the presence of an iron chelator by a CAS assay [19]. Cultures with a positive assay were centrifuged (20 min at 6,000*g*) and the supernatants were collected, pooled, and acidified to pH 2 with concentrated HCl. The resulting precipitate was removed via centrifugation. The clear supernatant was extracted with ethyl acetate (100 mL, three times). The pooled ethyl acetate fractions were dried over $NaSO_4$, filtered, and the solvent removed. BB was precipitated from the resulting residue by addition of ether (50 mL) to yield 11 mg of pure product. Anal. Calcd (Found) for $C_{39}H_{42}O_{13}N_6{\cdot}H_2O$: C 48.60 (48.22); H 5.33 (4.93); N 8.72 (8.61). MS (FAB+): m/z (MH^+) calcd 883; found 883.

Ferric complex transport assays in *B. subtilis*

B. subtilis was grown in both iron-free medium and in iron-replete medium to the late exponential phase. Production of siderophores was evident via the CAS assay [19] after this time period. Then, the cells were washed, suspended in the iron-limited medium, and kept on ice until the transport assay. The resuspension was set to an OD_{600} of 0.62 ± 0.02, corresponding to a culture density of 5.04×10^8 cells per milliliter (determined with a hemocytometer). After incubation of the cells (9 mL) for 10 min at 37 °C, the transport assay was started by addition of ^{55}Fe siderophore (1 mL at 0.9 μM). Aliquots (1 mL) were removed at appropriate times, filtered through membrane filters (HAWP Millipore, 0.45-μm pore size), and washed with 10 mL of cold 0.1 M sodium citrate. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added (from 10 mM stock in methanol) to achieve a total inhibitor concentration of 1 mM. Filters were dried and 6 mL of liquid scintillation Ecolume (ICN) was added. The vials were shaken, stored for 12 h, and the radioactivity determined using the Packard scintillation counter. Data are presented by normalizing the bacterial cultures used in each experiment to 1 mL for 5.04×10^8 cells, as determined by the hemocytometer data.

Ferric BB transport assays in *C. glutamicum*

These were as described for *B. subtilis*, except that 1 mL of ferric BB was added to 10 mL of culture.

General preparation of metal complexes

BB was isolated from *B. subtilis* as described already; the syntheses of the other compounds are referenced later. The iron complexes were formed by mixing $^{55}FeCl_3$ (3.7 Ci/mmol, 5 μL, 19 nmol) and $FeCl_3$ (2.9 μL, 72 nmol) with the corresponding ligand (100 μL, 100 nmol) in a ratio of 0.9:1 and ddH_2O (92.1 μL). This solution was incubated at room temperature for 2 h, after which sodium phosphate (100 μL, 1 M, pH 7.4) and ddH_2O (700 μL) were added. Free iron was removed by centrifugation at 14,000 rpm for 1 min. One hundred microliters of this concentrated stock solution (91 μM Fe) was diluted with 9.9 mL of the iron-free medium to

yield 10 mL of the working ferric complex solution (9.1 μM Fe). The radioactive ferric siderophore working solutions were filtered twice through the membrane filters (HAWP Millipore, 0.45-μm pore size). The concentrations of the ferric complexes were quantified by determining the specific radioactivity.

Michaelis–Menten kinetics

Aliquots of ^{55}Fe siderophore were added to two sets of six vials and diluted with culture medium to yield a distribution of concentrations (5, 1.75, 1, 0.75, 0.5, and 0.05 μM). Upon addition of bacterial culture (0.8 mL, OD_{600} = 0.6, cells washed and resuspended in fresh iron-free medium) the aliquots were incubated at 37 °C for either 2 or 5 min. The cultures were filtered through membrane filters (HAWP Millipore, 0.45-μm pore size) and washed with sodium citrate (0.1 M, 10 mL). The filters were dried and 6 mL of liquid scintillation Ecolume (ICN) was added. The vials were shaken, stored for 12 h, and the radioactivity determined using the Packard scintillation counter. A differential between the two time points indicated that uptake was occurring and the initial rate was determined between the values recorded at 2 and 5 min. Analysis of the experimental data with Origin 6.1 yielded K_m and V_{max}.

Synthesis of siderophore analogs

Enterobactin, enantioenterobactin, Trenhopo, and Mebiscam (MBC) were synthesized as described by Meyer et al. [33] and Xu et al. [34]. The syntheses of trencam (TC) and 2,3-dihydroxybenzoyl glycine are described in [35], and those of Trenglycam (TGC), Trensercam (TSC), Trenglucam (TEC), Trenlyscam (TKC), Trenbalcam (TAC), Trenmecam (TMC), and Trenbutcam (TBC) are described in [36]. Trentam was synthesized by Rebecca Abergel by a published procedure [37]. Desferrioxamine B (DFO) was a gift from Salutar. The syntheses of TMC and TBC are similar to those previously described [36].

Results and discussion

Isolation of BB from *C. glutamicum* and *B. subtilis*

BB was previously reported as isolated from *C. glutamicum* by Budzikiewicz et al. [5]; however, the published purification method did not yield the desired siderophore from 10 L of cultured medium after 2 days of growth. At no point during the incubation period did *C. glutamicum* supernatant give a CAS positive result. Extraction and isolation of the supernatant via the published procedure did not yield BB as evidenced by ^{1}H NMR spectroscopy or mass spectrometry. Consequently, BB was isolated from *B. subtilis* by a modified procedure [7]; see "Materials and methods."

Incorporation of BB by *C. glutamicum* and *B. subtilis*

Siderophore-mediated uptake has yet not been explored in *C. glutamicum* and has only partially been explored in *B. subtilis* [24, 26, 28, 29, 38]. ^{55}FeBB mediated uptake was measured under a variety of conditions (in iron-starved medium, in iron-replete medium, and with addition of a metabolic inhibitor) in both organisms. Transport in *B. subtilis* is as expected for an organism that produces this siderophore in response to iron starvation (Fig. 2). This process is energy-dependent; the metabolic inhibitor CCCP diminishes the ability of *B. subtilis* to accumulate iron. The uptake profile for *C. glutamicum* shows that this organism does not incorporate FeBB under any conditions (data not shown). In *B. subtilis*, maximal transport is seen when the cells are grown in iron-deficient medium. Although cold cells (0 °C) cannot

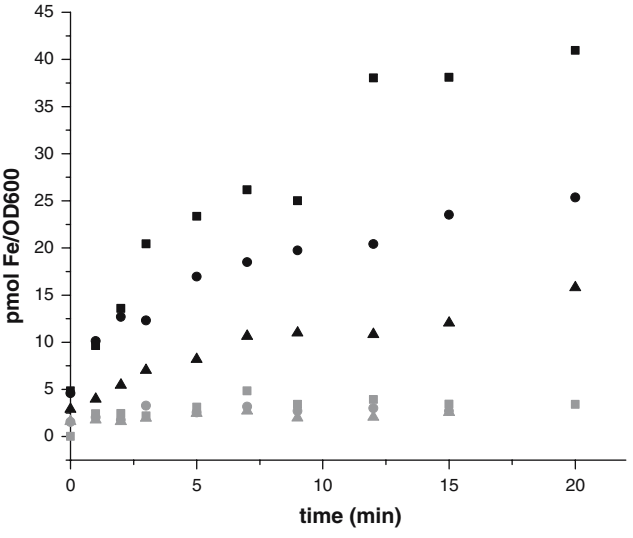

Fig. 2 Iron transport mediated by ^{55}FeBB (0.9 μM) in *B. subtilis* at 0 and 37 °C in iron-rich medium and iron-poor medium. Data presented are the average of two independent experiments. *Black squares* iron-poor media, 37 °C; *black circles* iron-poor media, 37 °C, metabolic inhibitor (carbonyl cyanide *m*-chlorophenylhydrazone, *CCCP*) added at 2.5 min; *black triangles* iron-poor media, 0 °C; *gray squares* iron-rich media, 37 °C; *gray circles* iron-rich media, 37 °C, metabolic inhibitor (CCCP) added at 2.5 min; *gray triangles* iron-rich media, 0 °C

transport the ferric siderophore complex, they have transporter systems expressed on the cell membrane since they were grown in iron-deficient medium. The differential between the uptake profiles of the two cell types (iron-deficient and iron-replete) at 0 °C is another indication of the presence of a siderophore transport pathway. No difference in the uptake profile is evident in any of the cells grown in iron-replete conditions.

Siderophore-related genes in *C. glutamicum* and *B. subtilis*

The recent completion of the sequencing of the genomes of *C. glutamicum* [31, 32] and *B. subtilis* [39] enables a search for siderophore-related genes. May et al. [7] identified the genes involved in the biosynthetic pathway for BB (*dhbABCEF*) in *B. subtilis* and found similarity between these genes and those responsible for enterobactin biosynthesis (*entABCEF*). A BLAST [40] search revealed that the BB biosynthetic genes of *B. subtilis* have very little similarity with genes from *C. glutamicum*. Of the five *dhb* genes, only one (*dhbC*) showed both sequence identity and a shared function to one gene in the *C. glutamicum* genome (*cg*1462, 37% identity at the amino acid level). DhbC is an isochorismate synthetase (ICS) and catalyzes the conversion of chorismate to isochorismate, which is an important siderophore or menaquinone precursor [41]. *Escherichia coli* and *B. subtilis* each contain two ISC isoenzymes. Although isochorismate is an early precursor in siderophore production, it can be incorporated into a variety of structurally different siderophores, so the presence of an ICS in *C. glutamicum* is not necessarily indicative of BB production [41]. Additionally, a recent publication employing both a biochemical and a bioinformatics approach to identifying the DtxR regulated genes in *C. glutamicum* did not find *cg*1462 [15]. Of the 26 genes found to be part of the iron metabolism of *C. glutamicum*, most are components of uncharacterized ABC transport systems. None are annotated as siderophore biosynthetic genes. Significantly, a BLAST search in the other corynebacterial species sequenced to date (*C. diphtheriae*, *C. efficiens*, and *C. jeikeium*) identified orthologs for EntA/DhbA, DhbC, DhbE, DhbF, and Fes (which encodes the esterase for the enterobactin trilactone ring) only in *C. jeikeium* [42]. Unfortunately, no reports have been published describing the production of a catecholate siderophore in this species. The absence of the required BB genes and the failure of iron-starved *C. glutamicum* to transport ferric BB indicate that *C. glucamicum* does not produce BB. This conclusion is in agreement with the observed CAS negative assay. For these reasons, we propose that the trimer of 2,3-dihydroxybenzoyl glycylthreonine should be called bacillibactin and that the original usage of ''corynebactin,'' referring to the uncharacterized siderophore of *C. diphtheriae*, should be restored.

Quentin et al. [43] inventoried the ABC transport systems of *B. subtilis*, while Baichoo et al. [44] used microarrays to determine the operons derepressed by both Fur mutations and iron starvation. More recently, Ollinger et al. [38] produced mutants to correlate Fur regulated genes with a particular siderophore. Ferrichrome and ferrioxamine each have a separate substrate binding protein (FhuD and YxeB, respectively), but require the same membrane permease using an ATPase (FhuBGC) [38, 45]. YfiYZ/YfhA/YusV is critical for growth with schizokinen and arthrobactin as iron sources [38]. Citrate is the substrate for another transporter (YfmCDEF), and growth stimulated by elemental iron requires YwbLMN [38]. *B. subtilis* requires FeuABC/YusV for growth when provided with ferric BB and enterobactin [38]. YuiL has homology with IroE protein of *Salmonella enterica*, which hydrolyzes enterobactin upon export [44, 46, 47]. YclQNOP has homology with FatD of *Vibrio anguillarum* [44], which is responsible for the transport of anguibactin, a catecholate siderophore, but a mutation of the yclN operon in *B. subtilis* did not generate a phenotype [38]. The only siderophore biosynthetic pathway found to date is that of BB, which has very high homology to the enterobactin biosynthetic pathway [7].

Siderophore-mediated iron transport in *B. subtilis*

Although the early studies investigated phenolate-mediated iron transport, some experimental issues obscure the significance of the results. First, citrate was present in the culture medium as a buffer [24]. As an α-hydroxycarboxylate, citric acid can act as a iron chelator; in fact YfmCDEF is the ferric citrate receptor in *B. subtilis* [38]. Second, the ferric complexes were made in situ, which could result in the complicated kinetics since the formation of the ferric complexes would equilibrate over the course of the uptake experiment [29]. Third, unwashed cells were used for the iron-incorporation experiment, making it impossible to determine exact amounts of 2,3-dihydroxybenzoyl glycine in the medium because it was both added by the investigators and produced by the organism. These early studies are still useful and correct in their descriptions of the generalities of iron transport, such as the energy and temperature depen-

dence of the transport, but are not useful in the determination of the specificity of the permease.

B. subtilis transports enterobactin and enantioenterobactin through the same permease as BB, evident from previous studies where an excess of nonradiolabeled ferric enterobactin (or enantioenterobactin, the mirror image of enterobactin) can block the incorporation of radioactive ferric BB [48]. The presence of an additional catecholate receptor, however, is inferred from the inability of nonradioactive ferric BB to block completely the incorporation of either radioactive ferric enterobactin or ferric enantioenterobactin even with increasing concentrations of ferric BB. The different chiralities of enterobactin and BB are not the root cause of the permease discrimination since the synthetically prepared mirror image of enterobactin, enantioenterobactin, has the same transport properties as enterobactin. The larger size of BB, rather than chirality, is the discriminating factor at the second receptor [48]. This result is reminiscent of the *S. typhimurium* siderophore-mediated pathway, where BB requires the IroN receptor in *S. typhimurium* and cannot be incorporated by FepA [49]. Molecular models of BB suggest that, compared with enterobactin, a more oblate ferric complex is formed, which may block receptor recognition [50]. *S. enterica* also expresses three catecholate receptors: Cir, FepA, and IroN [51]. Discrimination between enterobactin and BB is seen at the outer membrane, but the final entrance into the cell appears to take place at the same inner-membrane permease, FepDG [52]. Ollinger et al. [38] report the requirement for two lipoproteins for the hydroxamates ferrichrome and ferrioxamine in *B. subtilis*. Although only one receptor was indicated to be crucial for growth on either ferric enterobactin or ferric BB [38], the deletion of the gene responsible for the transport of ferric enterobactin only would not show any phenotype since this siderophore would still be able to be transported through the primary permease responsible for transporting both BB and enterobactin.

Pirating of exogenous siderophores is very common in the microbial world, and organisms often express receptors for siderophores other than their own. Transport of hydroxamates such as ferrichrome, ferrioxamine, and schizokinen through the FhuBCD transporter has been described previously [38, 45]. Although not natural siderophores, terephthalamides (TAM, see Trentam in Fig. 3) are derivatives of catecholamides. Use of TAM chelating moieties in place of catecholamide units in metal chelation drugs provides a compound with metal stability properties comparable to those of catecholamide compounds, but without the problem of oxidation of the aromatic ring [53]. Utilization of TAM-based drugs by microorganisms could present a potential problem for clinical use in treatment of iron overload, since recognition of the ferric-TAM complex could provide the organism with another avenue for iron acquisition. Both FeTrentam and FeDFO are incorporated into *B. subtilis*, and do not appear to be transported through the same permease as BB (Figs. 4, 5). Significantly, although

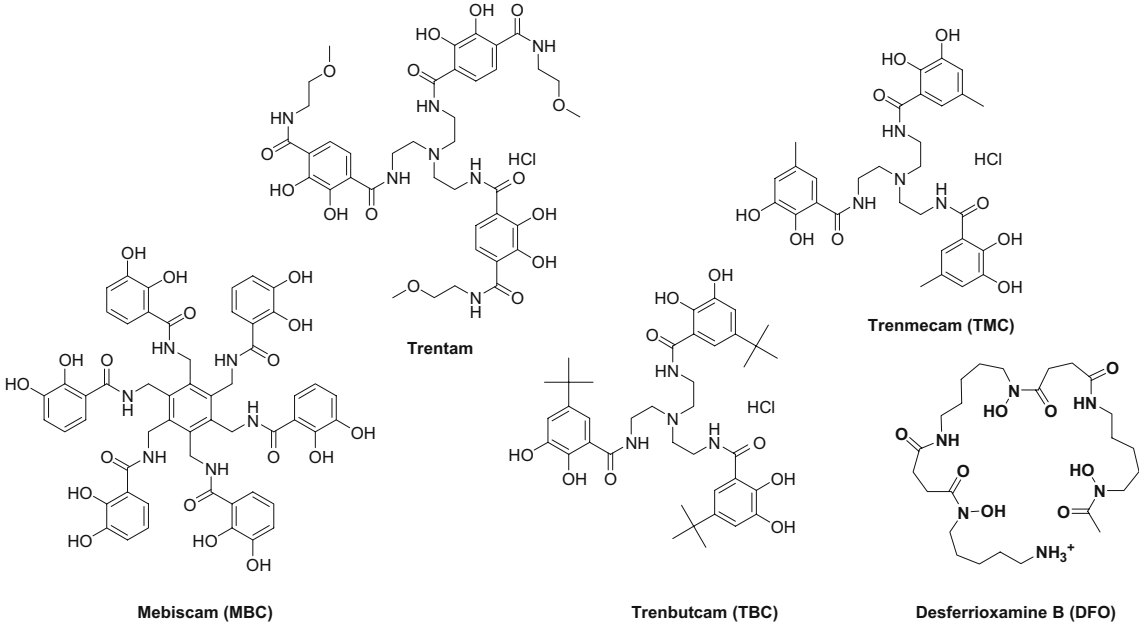

Fig. 3 Substituted catecholamide analogs, desferrioxamine B (*DFO*), and the diferric chelator Mebiscam (*MBC*) for uptake studies

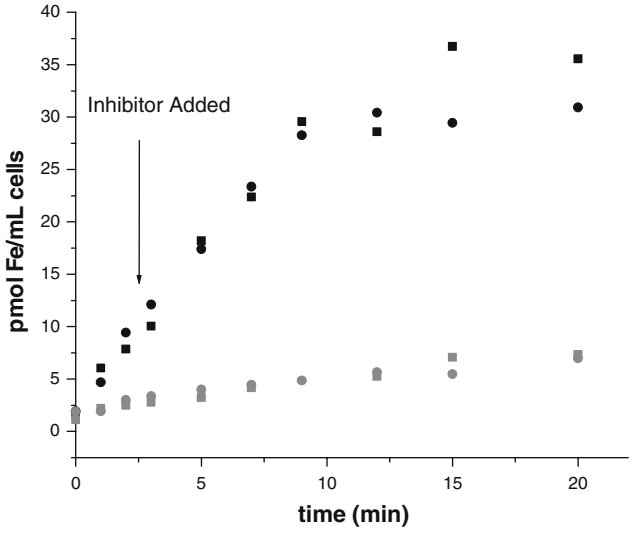

Fig. 4 Iron transport mediated by DFO (0.9 μM) in *B. subtilis.* Data presented are the average of two independent experiments. *Black squares* ^{55}FeDFO; *black circles* ^{55}FeDFO with cold FeBB (15-fold excess) added at 2.5 min; *gray squares* ^{55}FeBB; *gray circles* ^{55}FeBB with cold FeDFO (15-fold excess) added at 2.5 min

addition of nonradioactive FeDFO does not affect the incorporation of radioactive ferric BB, addition of nonradioactive FeTrentam does seem to affect the transport of ^{55}FeBB (Fig. 5). The reduction of intake of ^{55}FeBB, however, is not immediate, suggesting that the Trentam could be recognized by the BB permease,

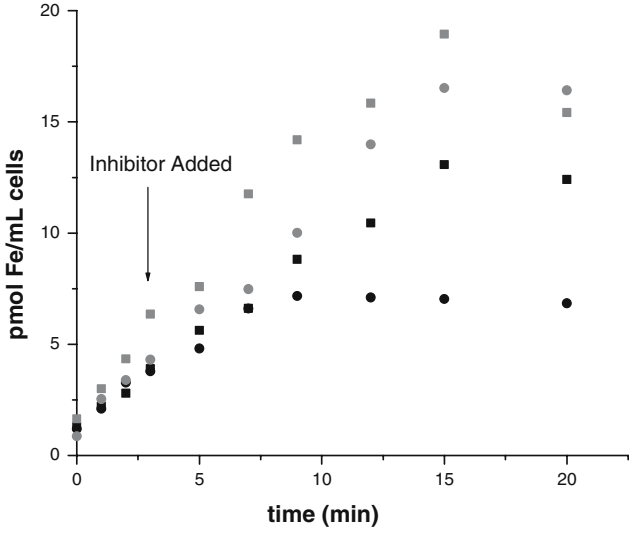

Fig. 5 Iron transport mediated by Trentam (0.9 μM) in *B. subtilis.* Data presented are the average of two independent experiments. *Black squares* ^{55}FeBB; *black circles* ^{55}FeBB with cold FeTrentam (15-fold excess) added at 2.5 min; *gray squares* ^{55}FeTrentam; *gray circles* ^{55}FeTrentam with cold FeBB (15-fold excess) added at 2.5 min

but with a much lower affinity (Fig. 5). Growth-promotion experiments are required to determine if *B. subtilis* can utilize the iron provided by Trentam.

The outer-membrane receptor, FepA, of *E. coli* does not recognize the backbone of enterobactin [54]. Consequently, synthetic analogs utilizing Tren or mesitylene backbones can be incorporated into the cell through this channel [55]. The size constraints imposed by the *B. subtilis* permease were studied through the design of catecholate analogs of BB and were of two general types: either steric bulk added to the 4- or 5-position of the catechol ring (Fig. 3, TMC or TBC) or insertion of amino acid spacers (Fig. 6, TAC, TEC, TGC, TKC, TSC) between the catechol and the Tren backbone. These seven new analogs can be compared with the original enterobactin analog, TC (Fig. 6). Pushing the size constraints to the maximum, MBC was also investigated. Addition of a 2,3-dihydroxybenzoyl moiety to each position of a mesitylene backbone provides a ligand with two potential iron binding sites (Fig. 3). Of the amino acid insertion analogs tested, all but ^{55}FeTEC and ^{55}FeTKC are transported at levels similar to that of ^{55}FeBB (Fig. 7). These two complexes, however, do inhibit ^{55}FeBB uptake (Fig. 8). Inspection of the ability of nonradioactive ferric Tren-based analogs (TEC, TKC, TSC, TAC, TGC, and TC) to block transport of ^{55}FeBB indicates that even Fe-TEC and FeTKC can block incorporation as effectively as the amino acid analogs that are actually transported. Ferric TEC and TKC may bind the permease, but transport is greatly hindered owing to either the larger size or the protonation constants of the amino acid side chains of both TKC and TEC, which cause these complexes to have an overall charge different from that of all the other analogs (FeTKC is neutral and FeTEC has a 6– charge overall, all others are 3–). TMC and TBC both contain alkyl substitutions at the 5-position of the catecholate ring, but do not contain an amino acid spacer between the catechol ring and the Tren backbone. Incorporation of ^{55}FeTMC and ^{55}FeTBC is evident from the difference in radioactive iron accumulation at 0 and 37 °C (data not shown). Methylation of the 5-position impedes the incorporation slightly, but TMC still delivers iron to the cell and can compete for binding to the FeBB receptor, while addition of the *tert*-butyl moiety greatly impedes incorporation (Fig. 9). Competition for the FeBB receptor is slight; addition of 15-fold excess nonradioactive FeTBC only marginally diminishes ^{55}FeBB incorporation. In the complementary competition experiment, where ^{55}FeTMC or ^{55}FeTBC is chased with nonradioactive FeBB, inhibition is not apparent. Similar to FeTKC or FeTEC, FeTMC and FeTBC may

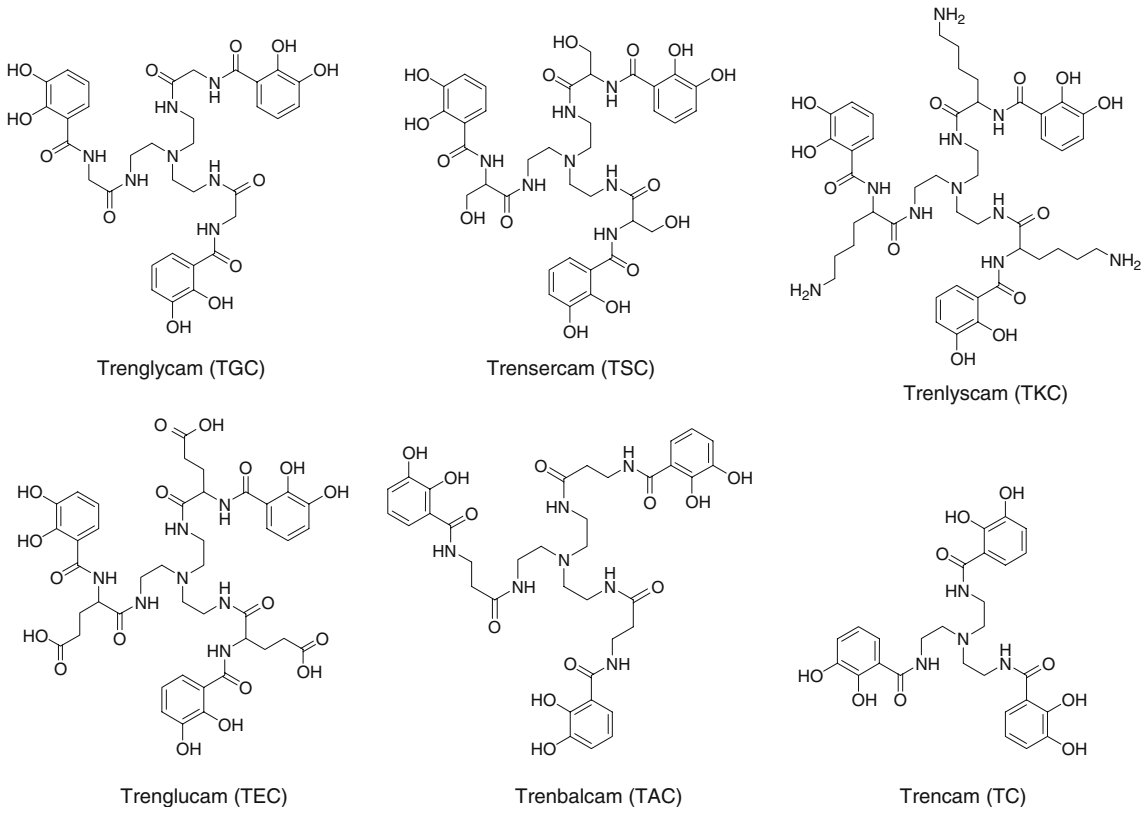

Fig. 6 Tren-based analogs

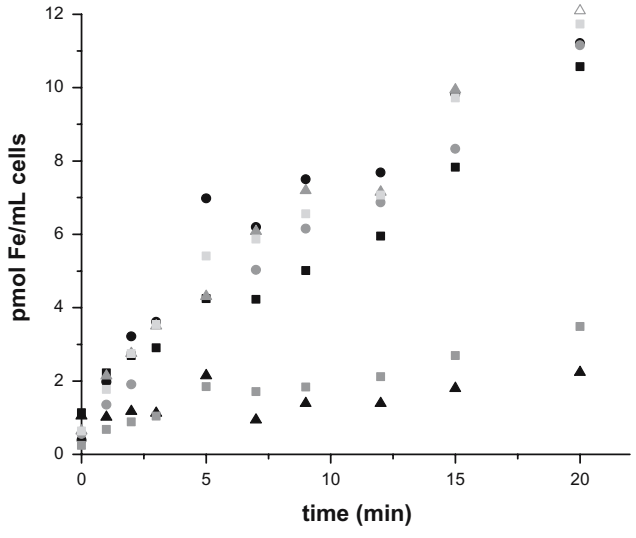

Fig. 7 Iron transport mediated by Tren-based analogs (0.9 μM) in *B. subtilis.* Data presented are the average of two independent experiments. *Black squares* ^{55}FeBB; *black circles* ^{55}FeTGC; *black triangles* ^{55}FeTEC; *dark gray squares* ^{55}FeTKC; *gray circles* ^{55}FeTSC; *gray triangles* ^{55}FeTC; *light gray squares* ^{55}FeTAC

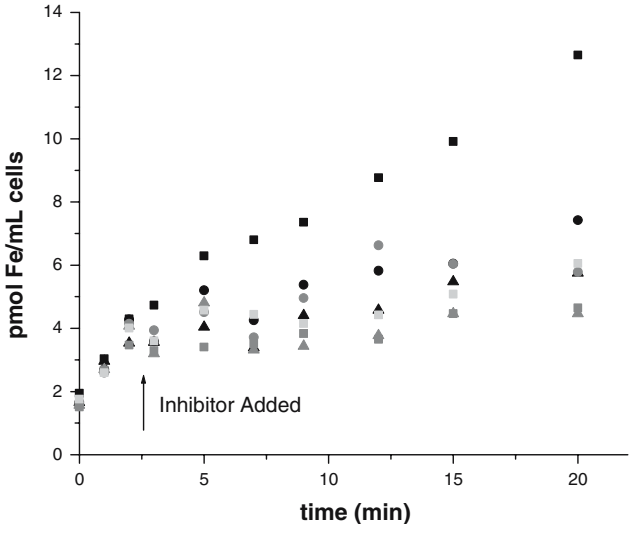

Fig. 8 Iron transport mediated by BB (0.9 μM) in *B. subtilis.* Data presented are the average of two independent experiments. *Black squares* ^{55}FeBB; *black circles* ^{55}FeBB with cold FeTSC (15-fold excess) added at 2.5 min; *black triangles* ^{55}FeBB with cold FeTEC (15-fold excess) added at 2.5 min; *dark gray squares* ^{55}FeBB with cold FeTAC (15-fold excess) added at 2.5 min; *gray circles* ^{55}FeBB with cold FeTKC (15-fold excess) added at 2.5 min; *gray triangles* ^{55}FeBB with cold FeTGC (15-fold excess) added at 2.5 min; *light gray squares* ^{55}FeBB with cold FeTBB (15-fold excess) added at 2.5 min

bind to the permease, but cannot be transported efficiently owing to steric constraints imposed by the channel. FeMBC provides two ferric ion chelation sites

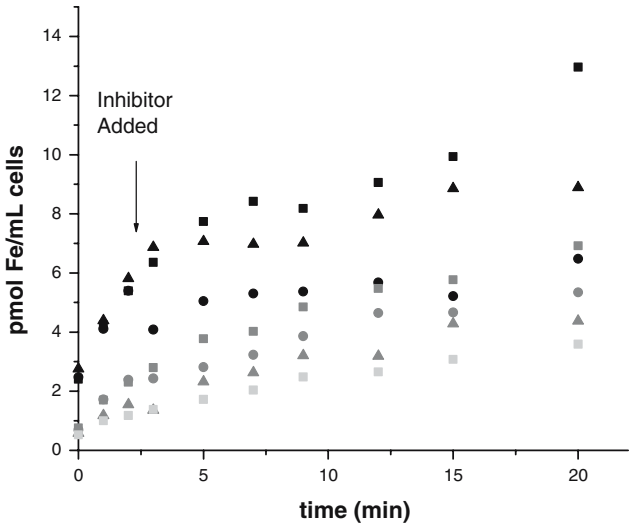

Fig. 9 Iron transport mediated by BB, TBC, and TMC (0.9 μM) in *B. subtilis*. Data presented are the average of two independent experiments. *Black squares* ^{55}FeBB; *black circles* ^{55}FeBB with cold FeTMC (15-fold excess) added at 2.5 min; *black triangles* ^{55}FeBB with cold FeTBC (15-fold excess) added at 2.5 min; *dark gray squares* ^{55}FeTMC; *gray circles* ^{55}FeTMC with cold FeBB (15-fold excess) added at 2.5 min; *gray triangles* ^{55}FeTBC; *light gray squares* ^{55}FeTBC with cold FeBB (15-fold excess) added at 2.5 min

and forms complexes with Fe_2L stoichiometry, mimicking the shape of tetradentate siderophores that form Fe_2L_3 complexes, but employing only one ligand rather than three. ^{55}FeMBC delivers more iron per cell than any of the other analogs tested. FeMBC appears to

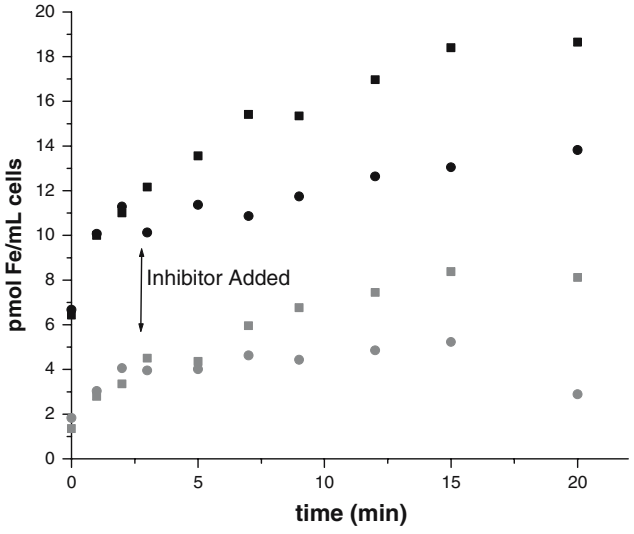

Fig. 10 Iron transport mediated by MBC (0.9 μM) in *B. subtilis*. Data presented are the average of two independent experiments. *Black squares* ^{55}FeMBC; *black circles* ^{55}FeMBC with cold FeBB (15-fold excess) added at 2.5 min; *gray squares* ^{55}FeBB; *gray circles* ^{55}FeBB with cold FeMBC (15-fold excess) added at 2.5 min

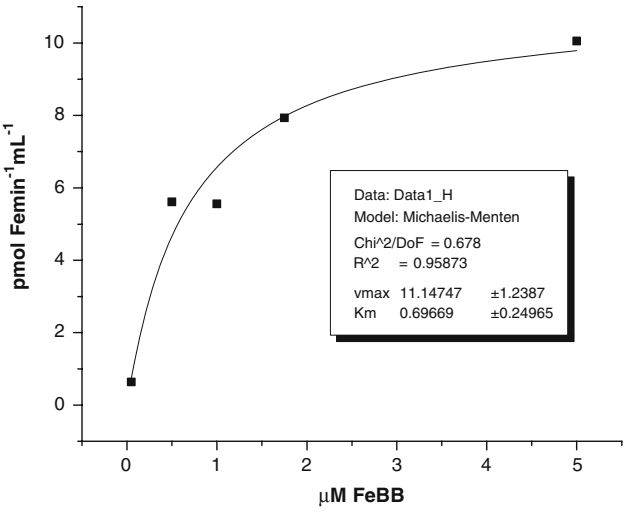

Fig. 11 Michaelis–Menten kinetics for FeBB. Data presented are the average of three independent experiments

enter the cell through the FeBB permease, since ^{55}FeMBC is effectively blocked by FeBB and vice versa (Fig. 10).

Rate experiments

Qualitative binding experiments were performed with the ferric complexes of BB, enterobactin, TKC, and DFO. Two time points (at 2 and 5 min) were taken and a differential between radioactivities indicated that transport rather than just binding was occurring. The endogenous siderophore of *B. subtilis*, FeBB, had the lowest K_m (0.7 μM, Fig. 11). Fe enterobactin is bound to the permease with a lesser K_m affinity, FeDFO has an affinity comparable with that of its permease. FeTKC shows the weakest interaction, but is still able to bind.

Summary and conclusion

These studies indicate that *B. subtilis* can incorporate a variety of ferric complexes via several parallel pathways. Replacement of glycine with another neutral amino acids does not affect the recognition, indicating that the primary recognition point is the triscatecholate ferric center. Replacement of the neutral side chain with charged moiety (glutamic acid and lysine) drastically diminishes the incorporation. All the Tren-based amino acid analogs can block ferric BB incorporation, despite the differences in size and charge of the resulting ferric complexes. This indicates that alteration of size or charge prevents the incorporation of the ferric complex into the cell, but not the binding of

Table 1 Summary of Michaelis–Menten kinetics

Ferric complex	K_m (μM)	V_{max} [pmol/(mL min)]
Bacillibactin	0.7 (.2)	11.1 (1.2)
Enterobactin	1.7 (.5)	27.5 (3.6)
Trenlyscam	7.7 (3.2)	13.1 (3.7)
Desferrioxamine B	3.0 (1.6)	9.7 (2.7)

The standard error is given in *parentheses*

the ferric complex to the permease. Indeed, though the K_m for FeTKC is higher than that of FeBB, FeTKC still can bind to the BB permease and block transport of BB if it is present in excess (Table 1). The *B. subtilis* catecholate permease tolerates some slight catecholate ring modification, in that FeTMC is incorporated, but at a substantially reduced rate, in contrast to FepA of *E. coli*, where a completely unsubstituted ring is required for recognition [55]. Transport of the diferric MBC complex is through the FeBB receptor, indicating that size does not matter as much as the charge and shape of the ferric complex.

Acknowledgements Lisa Whitworth assisted in maintenance of the bacterial cultures, and we thank John Helmann, Rebecca Abergel, and Trisha Hoette for many helpful discussions. This work was supported by NIH grant AI 11744.

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