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All organisms, including nearly all bacteria, require iron because of its important function in enzymes and its ability to bind oxygen. Iron is used in enzymes because of its ability to change oxidation state with only one electron difference between the two states, thus it is easily reduced or oxidized (Bullen and Griffiths, 1999).

Iron is the fourth most abundant element on Earth (de Voss *et al.*, 1999). Even so, bacteria seem to have difficulty in acquiring it from the environment and the human body. In anaerobic conditions, free Fe^{2+} is fairly available. However, when iron is exposed to oxygen, it is quickly oxidized to Fe^{3+} and becomes insoluble at neutral (physiological) pHs. This makes the element very scarce in the environment (Escolar *et al.*, 1999). In humans, and all other mammals, iron is tightly bound by proteins so that the concentration of free iron in the body is next to zero (Beveridge and Doyle, 1989; Schubert *et al.*, 1999). Because of this scarcity of iron, bacteria have evolved numerous different methods of acquiring it from various sources (de Voss *et al.*, 1999).

Bacteria have developed these varied methods of acquiring iron because of its importance to the cell. Iron is needed for many things in aerobic or facultative anaerobic bacteria: ATP synthesis, electron transport, DNA replication and synthesis all require iron (Beveridge and Doyle, 1989). Iron is so important to bacteria that some will use up to 10% of the cell's available iron and energy to produce heme (Bracken *et al.*, 1999). Some bacteria, like *Aquaspirillum magnetotacticum*, use iron to orient itself in the environment (Beveridge and Doyle, 1989). Iron is also an important component in proteins such as enzymes, cytochromes, and oxygen-carrying proteins like hemoglobin (Bullen and Griffiths, 1999).

Iron deficiency in bacteria causes many problems. Growth can be partially, or even completely, inhibited by lack of iron (Weinberg, 1977). Some enzymes, like catalase (Bullen and Griffiths, 1999; de Voss *et al.*, 1999), will not work properly without iron, causing the cell to slowly poison itself as it grows. Pathogenic bacteria also need iron to be virulent in their host. If iron is scarce and the bacteria has no way to capture iron, a normally pathogenic bacteria usually cannot cause any damage to the host, regardless of the number of the bacteria in the body.

Because of this critical need for iron in bacteria and its scarcity in the host, many species have developed methods with which to acquire iron from the host or from the environment. There are two main methods used: one that requires a low molecular weight compound called a siderophore, and the other that directly binds various types of iron-carrying proteins such as transferrin or hemoglobin. It seems that all Gram negative systems, regardless of the source of iron (siderophores or the direct binding of iron-sequestering proteins) involve the periplasmic protein TonB, the accessory proteins to TonB, and an outer membrane receptor that is generally very specific for the iron source being used. This special receptor is needed since normal porins cannot accommodate ferric siderophores (Beveridge and Doyle, 1989). All systems are, of course, ATP dependent. Gram positive systems are not TonB dependent, but also require energy and receptors to transport iron across the membrane.

Siderophores are complex molecules that are classified into two different types, based on their structure: the hydroxamates, such as aerobactin, and the catechols, such as enterobactin. They are always synthesized under low-iron conditions (Beveridge and Doyle, 1989), and each type has a very high affinity for iron and can compete with the host's own proteins for the iron (Bullen and Griffiths, 1999).

Production of siderophores in aerobic bacteria is common. Most aerobic (or facultative anaerobic) bacteria will produce some sort of siderophore themselves or have the ability to utilize other species' (Escolar *et al.*, 1999). Once the siderophore is produced, it will bind iron captured from the host and bring it back to the bacteria. The siderophore-iron complex will bind to a receptor and be transported into the periplasm. From there, it must cross the periplasm and the cytoplasmic membrane to get into the cytoplasm. These processes require transport proteins and energy.

The direct binding system requires specific receptor proteins for molecules such as transferrin, hemoglobin, heme, or any other iron-binding protein that the bacteria can utilize. The iron must then be taken from the protein (in some situations, the iron is still bound to heme) and carried into the periplasm by means of a transport protein. The free iron or heme must then be taken into the cytoplasm by permeases in the cytoplasmic membrane.

Both Gram negative and Gram positive bacteria utilize these systems, but the exact method may be different because of the difference in cell wall structures between the two types of organisms. Gram negative organisms will be covered first, followed by the Gram positive examples.

In Gram negative and Gram positive organisms, siderophores are produced in the cell and then excreted, for no contact between the host iron-binding protein and the cell is needed. The siderophore is excreted into the environment, where it will "hunt" an iron-binding protein, such as transferrin, compete with the protein for the iron, and then take the iron back to the cell (Bullen and Griffiths, 1999). Any siderophore production can be shown by means of CAS, or chrome azurol S, agar (Schubert *et al.*, 1999).

One of the main siderophores is used by several genuses of Gram negative bacteria, but was discovered in enteric bacteria such as *Escherichia coli*. This siderophore, enterobactin (or enterochelin), like all other siderophores, is made under iron-stressed conditions. A single enterobactin molecule is used only once by the cell, since the molecule is cleaved to release the iron to the cell (Bullen and Griffiths, 1999).

There are a few genes involved in enterobactin biosynthesis, transport, and the release of the iron bound to the enterobactin molecule. There are seven enterobactin biosynthesis genes, called *entA-G*. These genes are located on the *E. coli* chromosome and may act as a multi-enzyme complex to synthesize and take in enterobactin (Bullen and Griffiths, 1999).

Another main siderophore in enteric bacteria is aerobactin. Many organisms can use both aerobactin and enterobactin. However, aerobactin has a lower affinity for iron than enterobactin. Unlike enterobactin, however, aerobactin is recycled. If a bacteria can use both siderophores, because of the ability to recycle aerobactin, the cell may only use enterobactin in times of extreme iron stress (Bullen and Griffiths, 1999).

Five genes were found that are used to produce and use aerobactin. These genes, called *iucA, iucB, iucC, iucD,* and *iutA*, are clustered into an operon that is controlled by the regulatory protein Fur (Bullen and Griffiths, 1999). By various methods, usually deletion of the gene, it was discovered for what each gene product was used (Beveridge and Doyle, 1989).

IucA and IucC are subunits of a synthetase for aerobactin, IucB is an acetylase, and IucD is an oxygenase. These four proteins are all involved in the synthesis of aerobactin at various steps. IucD, though, is closely associated with the cytoplasmic membrane and is probably a transport protein (Beveridge and Doyle, 1989).

E. coli, in addition to aerobactin and enterobactin, can utilize other siderophores synthesized by other bacteria, such as ferrichrome, which is not degraded after use (like aerobactin) and coprogen (Bullen and Griffiths, 1999). *E. coli* strains that don't have siderophore systems have also been used in transformation experiments to see if a siderophore made by another species can make that *E. coli* strain able to take up iron. In nearly all cases, this was so.

Once the siderophore has obtained iron, the siderophore brings the iron back to the cell and binds to a specific receptor on the cell's outer membrane. All (that have been found) siderophore receptors interact with TonB, which is a periplasmic protein and spans the periplasmic space. Accessory proteins to TonB, ExbD and ExbB, help to change the conformation of TonB, which then changes the conformation of the receptor. This allows the iron or iron-binding protein to be let into the periplasmic space (Bullen and Griffiths, 1999; Nicholson and Beall, 1999). The iron is then picked up by a periplasmic binding protein (PBP) and sent through the cytoplasmic membrane by way of a ATPase-dependent permease. Once the iron is in the cytoplasm, it is then cleaved from its complex (if there was any part of the siderophore attached, as in the case of enterobactin) and put to use (Bullen and Griffiths, 1999). Enterobactin-bound iron must be cleaved from the enterobactin molecule by an esterase called Fes (Schubert *et al.*, 1999). What happens to the iron after it has entered the cytoplasm is not very well understood (Zhu *et al.*, 2000), but is probably put to use immediately.

There are many different types of receptors in each different genus for the various siderophores. These receptor systems can generally be classified as a single-component system or a two-component system. Both types of systems are TonB-dependent (Lewis *et al.*, 1998).

Single component systems only require a TonB-dependent receptor on the outer membrane. Many Gram-negative bacteria that use siderophores use this system. Two-component systems require a TonB-dependent receptor and an accessory lipoprotein. These systems include those used by *Neisseria* species, as well as many others, for the uptake of transferrin (Lewis *et al.*, 1998).

FepA is the receptor for ferric enterobactin, while IutA is the aerobactin receptor in *E. coli*. FepA has three domains: the usual surface region that binds the ferric enterobactin, a β-barrel domain inside the outer membrane, and the TonB box in the periplasmic space that interacts with TonB. The other *fep* genes include the other proteins needed for iron transport. FepB is the PBP for enterobactin, FepD and G are transmembrane proteins, and FepC is the ATPase (Bullen and Griffiths, 1999).

For transport across the cytoplasmic membrane in *E. coli* for some other siderophores (such as ferrichrome (Beveridge and Doyle, 1989)), the PBP is FhuD, the transmembrane protein, FhuC, and another protein, FhuB, which is an integral membrane protein that has two halves. This structure of FhuB has been found to be common in many homologous proteins in different species. This family of proteins, called the "siderophore family," is common to many bacteria that use siderophore uptake systems (*fatC* and *D* in *Vibrio*, and *hitB* in *Haemophilus*) (Bullen and Griffiths, 1999; Groeger and Köster, 1998).

Other bacteria that produce siderophores include *Vibrio* species (those that cause cholera and some types of infections by raw shellfish) (Bullen and Griffiths, 1999), *Yersinia* species (those that cause diseases like the Black Death) (Schubert *et al.*, 1999), *Psuedomonas* species (which cause a variety of diseases) (Bullen and Griffiths, 1999), and *Legionella pneumophila* (which

causes Legionnaire's disease) (Liles *et al.*, 2000), among several others of special note. These are all virulent species in some way or another.

Vibrio species produce siderophores called vibriobactin and anguibactin. The outer membrane receptor of vibriobactin is called ViuA. Anguibactin and the proteins needed for its uptake are encoded by the pJM1 plasmid, which contains the genes *fatA-D*. There are six transcription units on the plasmid. Units I, IV, V, and VI are involved in the biosynthesis of anguibactin. III encodes the positive regulator AngR (TAF is also required for regulation, but is encoded separately), and II includes four genes: *fatA* is an outer membrane receptor for anguibactin, *fatB* is the PBP, and *fatC* and *D* are the transmembrane proteins for the cytoplasmic membrane (Bullen and Griffiths, 1999).

Yersinia species utilize the siderophore yersiniabactin (Ybt) and can also use enterobactin. Enterobactin uptake is encoded by the *fep* and *fes* genes that are very similar to those found in *E. coli*. These genes are carried on the plasmid pSI10 (Schubert *et al.*, 1999).

Pseudomonas aeruginosa use many different siderophores. It can use its own, pyochelin and pyoverdine, or other species', including aerobactin and enterobactin. The outer membrane receptor for the pyoverdine and pyochelin siderophores is called PupA, while the enterobactin receptor is called PfeA and is similar to the *E. coli* FepA. (Bullen and Griffiths, 1999). An unusual method of uptake is also used by *P. aeruginosa*. *Myo*-inositol hexakisphosphate ($InsP_6$) has been shown to also facilitate the transport of iron into *P. aeruginosa* by way of a porin (the exact role of $InsP_6$ in some cells is still under debate) (Hirst *et al.*, 1999). In this sense, $InsP_6$ is acting like a siderophore. Usually, the best system is the only one used, since multiple systems are wasteful for the cell (Bullen and Griffiths, 1999).

Bordetella species also use a siderophore called alcaligin. The biosynthesis is iron controlled and is encoded by the *alcABCDER* operon. AlcA, AlcB, and AlcC are involved in the biosynthesis of alcaligin and have similarities to the *E. coli* proteins IucD, IucB, and IucC. The other genes are also involved in biosynthesis in some way, but for most, the exact way is still unknown. The *alcR* product is involved in regulation of the synthesis of alcaligin and the transport of the ferric alcaligin molecule. *alcR* seems to be related to the *Yersinia* gene *ybtA*, which is involved in yersiniabactin production. The outer membrane receptor is encoded by a gene located near the alcaligin operon and is called *fauA* (Brickman and Armstrong, 1999). *Bordetella* can also use other siderophores, such as enterobactin (receptor BfeA) (Nicholson and Beall, 1999).

Perhaps the least known siderophore is legiobactin, which is produced by *L. pneumophila*. It used to be thought that *L. pneumophila* did not require siderophores since it could live inside the cell and would have easy access to iron. However, some new studies have shown that *L. pneumophila* does live outside the cell, and would have a need of iron. It was also shown that there is siderophore activity in *L. pneumophila*. Iron is needed by this bacteria for "extracellular replication, intracellular infection, and virulence" (Liles *et al.*, 2000). This siderophore is unusual because it is not a catecholate or a hydroxamate as most other siderophores are, though a hydroxamate synthase-like protein (FrgA) has been found in this genus. This caused a problem in the detection of legiobactin by the usual methods (CAS, Arnow, and Csáky methods) (Liles *et al.*, 2000).

However, sometimes legiobactin is not produced in some *L. pneumophila* colonies. It seems that once a population has passed the late stationary phase of growth, they cannot make

any siderophore, even if iron becomes scarce. And colonies that do make legiobactin will no longer make it after this point in late stationary phase, even if iron is still scarce. Perhaps this is because iron is no longer needed in this stage of growth (Liles *et al.*, 2000).

From the above examples, it can be concluded that there are many different types of siderophores produced by many different genera. It also seems that important disease-causing organisms are the ones that have multiple siderophores that they can utilize, but all have at least one siderophore. As will be seen next, siderophores are not the only way the cell can take up iron. However, many of the following bacterial examples may also use siderophores as well as these different methods. Like the siderophore uptake methods, the following methods are TonB dependent and require energy.

Siderophores are not the only way a cell may take up iron. Iron itself, when plentiful (and not in the Fe^{3+} state), may just be pumped across the membrane by a permease that requires ATP. Other sources of iron other than that attached to iron-binding proteins may also be utilized. Ferric citrate is an important iron source for *E. coli, Neisseria meningitidis* and *P. aeruginosa*. FecA is a TonB-dependent outer membrane protein that may take up ferric citrate as an iron source, since it only appears in bacteria growing in low iron media containing citrate. FecC and D are cytoplasmic proteins, and FecB is modified but does end up in a different form in the periplasm. FecE may be an ATPase, FecI and R are both positive additional regulatory proteins (only produced in the presence of citrate) (Bullen and Griffiths, 1999).

Heme can also be utilized by many different types of bacteria. Heme and iron-containing carrier proteins like hemoglobin, transferrin, and lactotransferrin are also used as iron sources for

Gram negative bacteria. Usually, the receptors for these molecules are extremely host specific (Bullen and Griffiths, 1999).

Unlike siderophore-dependent iron uptake, direct contact between the bacterial cell and the iron-binding molecule is needed. Specific receptors are needed for each type of molecule, though some receptors can take up similar molecules (like hemoglobin and the haptoglobin-hemoglobin complex) (Bullen and Griffiths, 1999).

There are two proteins that have been identified as transferrin receptors in many different species. Tbp2 or TbpB is a lipoprotein that also acts as an antigen to the host, and Tbp1 or TbpA is the receptor itself. TbpA does not seem to need TbpB to function, though it is TonB-dependent (Bullen and Griffiths, 1999).

Transferrin itself is not usually internalized by the cell, so the iron bound by the transferrin molecule needs to be removed and then brought into the cell (it should be noted that *Pseudomonas* does not need to cleave the iron from the transferrin, but it usually does in infection). In Gram negative organisms, the iron must then cross the periplasm and the cytoplasmic membrane. The cleaving of the iron from the transferrin is carried out by a protease, which disrupts the iron-binding site and allows iron to be released. This cleaving may be the cause of tissue damage in some diseases, such as cystic fibrosis. Another method of taking the iron away from the transferrin molecule is to reduce Fe^{3+} to Fe^{2+}, since transferrin will not bind Fe^{2+} (the Gram positive species *Listeria monocytogenes* has been shown to do this, so other types of bacteria might as well) (Bullen and Griffiths, 1999).

Neisseria meningitidis and *Neisseria gonorrhoeae* both use this direct binding uptake method of iron. Iron-sources of this type *Neisseria* can use include: transferrin, lactoferrin, heme,

hemoglobin, and the haptoglobin-hemoglobin complex (Lewis *et al.*, 1998). Of course, the uptake of these iron sources (after the iron has been cleaved from the molecule) is TonB-dependent.

N. meningitidis uses two main systems to utilize non-siderophore iron. The first is a two component system. The HpuAB system is made up of two proteins, HpuA and HpuB, and is TonB-dependent. HpuA is the lipoprotein and HpuB is the transport protein. This system binds and removes iron from hemoglobin and the hemoglobin-haptoglobin complex (Bullen and Griffiths, 1999; Lewis *et al.*, 1998). The other system is the HmbR single component system that binds and removes iron from hemoglobin. Meningococcal bacteria can use either or both of these systems, depending on the strain (Lewis *et al.*, 1998).

Heme is a toxic compound to most bacteria, but it is still needed by the cell. Because of this toxicity, the bacteria have a way to destroy the heme for its component parts so that it is no longer toxic to the cell. It is unlikely that bacteria take up the whole heme molecule and have some way of reducing or eliminating the toxicity of the heme molecule in any other way than the destruction of the molecule. Bacterial heme destruction is similar to human heme destruction in that a type of protein, called an oxygenase, is used to carry out the destruction of heme (Zhu *et al.*, 2000).

Some bacterial oxygenases are encoded by a gene called *hemO*, which was originally found in *Neisseria* species, or they use a similar gene. The HemO protein resembles human oxygenases. Mutations in this gene cause the cell to be unable to use hemoglobin or heme as an iron source, though other iron sources can still be used. This means that only the heme-related iron uptake was affected, and not the uptake of other iron sources. Colonies that contain HemO

are also colored by a byproduct of heme destruction, biliverdin, showing that truly, heme destruction is occurring in those colonies that contain HemO (Zhu *et al.*, 2000).

HemO expression is linked with HmbR expression. The more HemO is made, the more HmbR receptors are made as well. Conversely, if there is a mutation in *hemO* or if there is too much heme in the environment, the expression of HmbR is reduced. This would mean that these two genes are linked transcriptionally; *hmbR is* located a little downstream from *hemO*, which supports this proposed linking of the genes (Zhu *et al.*, 2000).

The *Neisseria* species are not the only species that use heme and heme-related compounds for iron sources. In *Yersinia pestis*, heme seems to be the most efficient source of iron. *Y. pestis* can also use hemopexin, myoglobin, haptoglobin, and hemoglobin as iron sources (Bullen and Griffiths, 1999; Schubert *et al.*, 1999). The uptake of heme in any form is dependent on the products of the *hmu* gene locus (Bullen and Griffiths, 1999) and the heme or heme-containing molecule receptor HemR (Bracken *et al.*, 1999). The HemR receptor was compared to the *Neisseria* species HmbR heme receptor, and it was found that HemR allowed the bacteria to use many more different sources of heme than the HmbR receptor. The HemR receptor could accept heme, hemoglobin, myoglobin, hemopexin, and catalase (though heme and hemoglobin were the best sources of heme), while HmbR could only accept heme and hemoglobin. HemR is, of course, a TonB dependent receptor (Bracken *et al.*, 1999).

Y. pestis can also store heme, which may play a role in the speed and severity of the onset of disease (to be covered later). Storage depends on the four *hms* genes: *hmsH, hmsF, hmsR,* and *hmsS*. The exact functions of the gene products are not yet known (Bullen and Griffiths, 1999).

Haemophilus influenzae can also use hemoglobin, heme-hemopexin, and hemoglobin-haptoglobin as iron and heme sources, since it cannot directly take up heme (Bullen and Griffiths, 1999). Hemoglobin and the hemoglobin-haptoglobin complex is bound by a receptor designated HgpA, and hemoglobin-haptoglobin alone is bound by HhuA. *H. influenzae* also has an iron-binding system of *hit* gene products; HitA is thought to be a periplasmic iron-binding protein, HitB a cytoplasmic permease, and HitC a type of ATPase (Adhikari *et al.*, 1995).

Heme can be internalized by either bringing in the entire heme-receptor complex, or by having the heme cleaved and transferred to another protein to be internalized. If the heme-receptor complex is brought into the cell, the heme would be cleaved from the receptor shortly after internalization (Bullen and Griffiths, 1999). After the iron has been cleaved from the iron-binding molecules, or the entire molecule has been internalized (in the case of *H. influenzae* with heme (Bullen and Griffiths, 1999)), it must be transported across the periplasm and the cytoplasmic membrane to the cytoplasm. This method is probably very similar to the siderophore-dependent systems where iron (or in some cases, the heme molecule) is bound by a periplasmic binding protein and carried to a cytoplasmic permease that is ATP dependent (Bullen and Griffiths, 1999; Adhikari *et al.*, 1995).

These different methods of iron-uptake from many different sources is important for virulence of the Gram negative species mentioned. The more methods of uptake they can use, they more likely the bacteria are to get the iron they require. But Gram negative species aren't the only ones that require iron.

Gram positive bacteria must also take up iron to survive. They can use both siderophore-independent and -dependent methods of iron uptake. Because Gram positive bacteria do not have

an outer membrane or a periplasmic space, the method of uptake has to be different from that of Gram negative bacteria. There is no TonB-dependent uptake (since TonB is a periplasmic space-spanning protein), but there still must be an energy source and a receptor to bind and absorb iron (Bullen and Griffiths, 1999).

Staphylococci are a Gram positive bacteria than can use both siderophore-dependent and -independent iron uptake (Bullen and Griffiths, 1999). But since *S. aureus* seems to have a low requirement for iron, it was awhile before any siderophores were discovered in this species. Recently discovered was the *S. aureus* siderophore called aureochelin. *S. hyicus* has also been shown to use two siderophores, staphyloferrins A and B. The SirA protein might be a receptor protein for these staphyloferrins. The receptor and siderophore production is probably controlled by a Fur-like protein, as some Fur-box-like fragments of DNA have been found (Heinrichs *et al.*, 1999). *S. aureus* has also been shown to use enterobactin (Courcol *et al.*, 1997) and sometimes the staphyloferrins (Heinrichs *et al.*, 1999), however, the majority about how *S. aureus* takes up iron from media is still mostly unknown (Courcol *et al.*, 1997).

Mycobacteria (they will be considered Gram positive in this instance) also use siderophores. Mycobacterial siderophores are classified as mycobactins (MBs) or exochelins. These siderophores, like those produced by Gram negative organisms, are made when the concentration of iron in the medium is very low. These siderophores also seem to have a high affinity for iron, just like those of the Gram negative organisms (de Voss *et al.*, 1999).

Each type of siderophore seems to have two different types of their own. One type is associated with the cell and may act as a receptor or transporter, and the other is secreted and actually captures the iron from the host's iron-binding proteins. The extracellular mycobactins

have been shown to acquire their iron from transferrin and then transfer it to the cell-associated mycobactins (de Voss *et al.*, 1999).

The genes that encode the proteins that assemble mycobactins are clustered together and called *mbt*. The proteins encoded by these genes are similar to those in other bacteria that produce siderophores. If one of these genes is mutated, no siderophore is produced, meaning that all types of mycobactins are produced through a common pathway. This mutant was also avirulent, showing that iron is also needed for infection by mycobacteria (de Voss *et al.*, 1999).

Siderophore-independent mechanisms of iron uptake are also known in many Gram positive bacteria. *Staphylococcus aureus* can use transferrin as an iron source; its binding protein seems to be homologous to the glycolysis enzyme glyceraldehyde-3-phosphate dehydrogenase (Bullen and Griffiths, 1999).

Mycobacteria can also use salicylic acid and citric acid, as well as transferrin itself. A high concentration of salicylic acid is secreted into the medium when the bacteria is grown under low iron concentration conditions. It is unsure whether salicylic acid is used as a siderophore or whether it is used to solubilize iron so that it can be taken up by the mycobactins and exochelins (de Voss *et al.*, 1999).

Heme is also used by some Gram positive bacteria, such as *S. aureus, Streptococcus faecalis* and *Bacillus cereus*. As with Gram negative bacteria, heme is toxic to Gram positive cells, so a way of breaking down the heme is needed. There is a oxygenase in Gram positive bacteria, HmuO, that acts like HemO in Gram negative bacteria. HmuO was the first prokaryotic heme oxygenase to be discovered; it was discovered in *Corynebacterium diptheriae* and *C. ulcerans* (Zhu *et al.*, 2000).

Uptake of iron by all these systems must be carefully controlled. Too much iron can cause bacteria problems, such as causing breaks in the DNA of an organism or killing it (Bullen and Griffiths, 1999), just as much as too little iron can, since iron is needed for so many different things in the cell. However, excretion methods have never been found in bacteria (Escolar *et al.*, 1999). For the most part, *all* iron uptake seems to be negatively controlled by a special iron-binding protein commonly called Fur (mentioned earlier), for ferric uptake regulation (Beveridge and Doyle, 1989), but may also be controlled additionally by other systems (Bullen and Griffiths, 1999). Other genuses of bacteria may have similar proteins under a different name, but the method of regulation is the same (Escolar *et al.*, 1999). It should be known that for the examples of siderophore activity given in many different genuses, the genes that produce these siderophores are *all* controlled by Fur or a homologous protein.

This regulation occurs at the transcriptional level, meaning that Fur is a DNA binding protein. Iron (that is, Fe^{2+}, since Fe^{3+} cannot be used with Fur (Hamza *et al.*, 1999)) is an important co-repressor to the Fur protein (Beveridge and Doyle, 1989). Iron will bind to Fur when iron is plentiful, causing Fur to bind to the DNA at the segment called the "iron box" or "Fur box". When the DNA is bound by Fur, polymerases cannot transcribe the genes. Once iron is scarce, the iron binding Fur will be released, causing Fur to "fall off" of the DNA, and then the genes can be transcribed (Escolar *et al.*, 1999).

Fur regulates many genes that have to do with iron uptake (such as siderophores and heme receptors), as well as those genes that are involved in metabolic functions that are controlled by iron. Fur regulates chemotaxis, some metabolic pathways, the defense against oxygen radicals, and the production of toxins and virulence factors. Fur is also a very abundant protein in the cell.

In the log phase of bacterial growth, there has been an estimation of over 2,500 molecules of Fur in one cell. This can increase to 7,500 molecules in the stationary phase. This abundance of Fur is probably related to its wide use for regulation in the cell (Escolar *et al.*, 1999).

FURTA (Fur titration assay), as well as the SELEX (systematic evolution of ligands by exponential enrichment) technique, allows genes regulated by Fur to be identified. Sixteen different Fur-dependent genes have been recognized in *P. aeruginosa* alone by these techniques. Other techniques for identifying Fur-regulated genes include screening for manganese resistant mutant colonies, since manganese acts like iron in high concentrations. This causes a lethal repression of any iron uptake systems. If a colony does not have Fur, these systems are not repressed (Escolar *et al.*, 1999).

Other Fur-like proteins have been found in some genuses. The production of mycobacterial siderophores is controlled by a Fur-like protein called IdeR. Like Fur, IdeR binds both iron and DNA, showing that it is a iron-regulated DNA binding protein (de Voss *et al.*, 1999; Dussurget *et al.*, 1999). Other Fur-like proteins have been found in other Gram positive species such as *Bacillus subtilis*, *Staphylococcus* species, and cyanobacteria. *B. subtilis* also has Fur boxes in the DNA to which the Fur-like proteins can bind to regulate siderophore synthesis, among all the other processes that Fur regulates (Escolar *et al.*, 1999).

There may also be some other mechanisms of repression other than Fur or related proteins. This is usually a positive regulation method (many have already been cited) which involves the presence of another protein or an iron-containing compound like citrate (Bullen and Griffiths, 1999).

Because iron is so important in metabolic functions, bacteria have produced so many different types of iron-uptake systems. Iron uptake by these systems seems to have one more very important function. Bacteria that can uptake iron are also more virulent in the host than those that cannot uptake iron. There are many reasons why this is so.

Mammalian hosts, however, were not left unguarded against attack. There are many different bactericidal or bacteriostatic compounds in the plasma and tissues of mammals. These compounds generally keep bacteria from obtaining the iron they need so much to grow and multiply (de Voss *et al.*, 1999). Iron-binding proteins keep the plasmic iron concentration near to zero (about 10^{-18} M) (Bullen and Griffiths, 1999), so free iron is not easily obtained. Competition for this bound iron occurs between the iron-sequestering compounds of the host (like transferrin, lactotransferrin, ovotransferrin, and heme (Beveridge and Doyle, 1989)) and the siderophores of the bacterial invaders (Weinberg, 1977). Transferrin and haptoglobin by themselves can inhibit the growth of some organisms like *Mycobacterium tuberculosis* (de Voss *et al.*, 1999).

The presence of heme, which is the most abundant source of iron in the body (Zhu *et al.*, 2000), has been shown to increase the virulence of *E. coli*, and it is now known that it is the iron bound to the heme that is so important for growth (Bullen and Griffiths, 1999).

According to Weinberg, "the effectiveness of biological iron carriers in binding the metal and the stability of iron-carrier complexes are influenced by hydrogen ion concentration (pH), temperature, the presence of other iron-binding materials or exogenous iron, and the enzymatic disintegration of the carriers" (1977). Meaning that the ability of transferrin and other such molecules to hold onto their iron is based on factors such as the pH of the blood, body temperature, and whether or not they can be degraded by enzymes. The E_n, or the "oxidation

reduction potential" is also important in the action of bactericidal systems (Bullen and Griffiths, 1999).

The patient's iron status is another indication of how virulent a bacteria may be. A patient with too much or too little iron in their blood (especially if this is free iron) is more susceptible to infection for various reasons. Lymphocytes depend on iron bound to transferrin to be activated. If this iron is not present (because of the taking of the iron by bacteria or by some other means), the amount of lymphocytes produced is decreased dramatically (50-90%), leaving the patient ripe for infection. Blood transfusions in patients, like those with β-thalassaemia, also increase the iron available, as well as thalassaemia-caused splenectomies, which will also increase the risk of infection (Bullen and Griffiths, 1999).

Other molecules in the serum of mammals help to keep bacteria from obtaining iron. Serum, and therefore the molecules contained in it, can kill nearly all *E. coli* cells in a sample in two hours. Bacteria are especially sensitive to these bactericidal molecules if they are iron starved (Bullen and Griffiths, 1999).

Antibodies might also bind to the siderophore receptors of some bacteria or react with some other types of iron-regulated outer membrane proteins to keep them from accepting iron. Complement might also contribute to the bactericidal effect of antibodies and transferrin (Bullen and Griffiths, 1999).

Bacteria can fight against the host's defenses with siderophores and other iron uptake methods. These molecules can compete with the host's iron-binding proteins and "steal" the iron from the host (Escolar *et al.*, 1999). The ability of the siderophore to compete with the iron-binding molecules of the host directly relates to the virulence and the survival of the species that

produced the siderophore (Adhikari *et al.*, 1995; Weinberg, 1977). This stealing of the iron from the host's iron-carrying molecules can cause many problems, as those listed above if there is too little iron available, such as the decrease in lymphocyte production (Bullen and Griffiths, 1999).

Siderophores may also increase the virulence of some organisms by causing destruction and inflammation of the host's tissues by the formation of free radicals (common when iron and oxygen are involved) and suppressing the immune response of the host (Bullen and Griffiths, 1999).

Another factor that increases virulence is to increase the concentration of available iron. One way this is done is by the production of haemolysins, which destroy the red blood cells that carry heme. Injury, like a gun shot wound, can also cause the lysis of red blood cells. Injury can also spread bacteria to places where they don't belong (such as *E. coli* from the intestine carried into the peritoneal cavity by the rupture of the intestine by the above gun shot), and with all this iron suddenly available, infection can easily start (Bullen and Griffiths, 1999).

Haemolysin production is iron-regulated, and the genes for the production are found on the Hly plasmid. *E. coli*, *V. cholerae,* and *Serratia marcescens* all can produce haemolysins (Bullen and Griffiths, 1999).

Yersinia species, especially *Y. pestis*, which causes the Black Death, have a special way of making sure they get the iron they need to infect a host. When a flea takes a drink of blood, that blood is broken down and mixed up with the *Yersinia* bacteria that live in the gut of the flea. Here, the *Yersinia* have all the iron and heme they need. When the flea next bites a victim, some of the flea's stomach contents, heme and bacteria both, are injected into the host. The *Yersinia* bacteria now have a good source of iron without having to worry about obtaining it from the

host's iron-binding molecules, and can grow quickly to infect the host. This is the way *Yersinia* ensure their virulence (Bullen and Griffiths, 1999).

If these iron uptake systems are disrupted or somehow lost (as in the loss of a plasmid), then the virulence of many types of bacteria can decrease or even be lost. One example of this is the fish parasite *Vibrio anguillarum*, which gains an iron-uptake siderophore, anguibactin, from the plasmid pJM1. It has been found that when the plasmid is lost, the bacteria is no longer virulent in fish and cannot grow under low-iron conditions (Bullen and Griffiths, 1999).

Non-virulent strains that have the ability to accept the siderophore anguibactin but can no longer make it (it still has the FatA anguibactin receptor), can be kept alive by adding a virulent strain with a wild-type plasmid (meaning that it is making the siderophore as usual), showing that this plasmid, and the iron-uptake system it codes for, is they key to virulence in *V. anguillarum*. Also, *V. cholerae* produces a protein, IrgA, that when mutated, decreases the virulence of the bacteria, but not the growth. It was found that this protein was regulated by iron and possibly Fur (Bullen and Griffiths, 1999).

H. influenzae causes many different types of diseases, some that are not very dangerous. These less dangerous strains may lack virulence because they also lack the *hit* operon, and can no longer uptake iron as they should. The more virulent forms, then, have an intact *hit* operon and can easily cause disease (Adhikari *et al.*, 1995).

Iron concentration and the ability of the bacteria to uptake iron can be major factors in the danger of some diseases. Diseases that are affected by iron uptake include: diabetes, leukemia, sepsis, sickle cell anemia, and malaria. In some way or another, the availability of iron in those affected by these diseases is increased, causing the bacteria that cause these diseases to be more

virulent because they are now more resistant to the bacteriostatic and bactericidal systems of the plasma. If bacteria have plenty of iron, the host's defense systems crumbles (because of the decreased sensitivity to the bactericidal or bacteriostatic effects of plasma), allowing the bacteria to proliferate.

Because of iron's importance in virulence, studies have been started to see if siderophores can be used to combat infection by the bacteria that produce these siderophores. The idea is to substitute another heavy metal, such as Sc^{3+} or In^{3+} for iron in the siderophore, which has been shown to have a bacteriostatic effect (Bullen and Griffiths, 1999).

Perhaps knowing more about the iron uptake systems of numerous types of bacteria will help in eradicating the threat of virulent disease caused by such types of bacteria. If the uptake of iron is prevented, then the bacteria will likely die because of its inability to take up iron. But because there are so many different ways that a single genus could take up iron, this could be a daunting task. However, if these iron uptake systems can be understood and controlled, virulent bacteria will no longer be a problem.

Sources

Adhikari, Pratima, Shane D. Kirby, Andrew J. Nowak, Kristen L. Veraldi, Anthony B. Schryvers, Timothy A. Mietzner. "Biochemical Characterization of a *Haemophilus influenzae* Periplasmic Iron Transport Operon." The Journal of Biological Chemistry. Oct 1995: 25142-25149.

Beveridge, Terrance J. and Ronald J. Doyle, eds. Metal Ions and Bacteria. New York: John Wiley and Sons, 1989.

Bracken, Charles S., Michael T. Baer, Asiya Abdur-Rashid, Whitney Hemhs, Igor Stokiljkovic. "Use of Heme-Protein Complexes by the *Yersinia enterocolitica* HemR Receptor: Histidine Residues Are Essential for Receptor Function." Journal of Bacteriology. Oct 1999: 6063-6071.

Brickman, Timothy J. and Sandra K. Armstrong. "Essential Role of the Iron-Regulated Outer Membrane Receptor FauA in Alcaligin Siderophore-Mediated Iron Uptake in *Bordetella* Species." Journal of Bacteriology. Oct. 1999: 5958-5965.

Bullen, J.J. and E. Griffiths, eds. Iron and Infection. New York: John Wiley and Sons, 1999 edition.

Courcol, Rene J., D. Trivier, M. Bissinger, Guy R. Martin, M.R.W. Brown. "Siderophore Production by *Staphylococcus aureus* and Identification of Iron-Regulated Proteins." Infection and Immunity. May 1997: 1944-1948

de Voss, James J., Kerry Rutter, Benjamin G. Schroeder, Clifton E. Barry III. "Iron Acquisition and Metabolism by Mycobacteria." Journal of Bacteriology. August 1999: 4443-4449.

Dussurget, Olivier, Juliano Timm, Manuel Gomez, Benjamin Gold, Shengwei Yu, Sue Z. Sabol, Randall K. Holmes, William R. Jacobs, Jr., Issar Smith. "Transcriptional Control of the Iron-Responsive *fxbA* Gene by the Mycobacterial Regulator IdeR." Journal of Bacteriology. June 1999: 3402-3408.

Escolar, Lucía, Jose Pérez-Martín, Víctor de Lorenzo. "Opening the Iron Box: Transcriptional Metalloregulation by the Fur protein." Journal of Bacteriology. Oct. 1999: 6223-6227.

Groeger, Wolfram and Wolfgang Köster. "Transmembrane Topology of the two FhuB domains representing the hydrophobic components of bacterial ABC transporters involved in the uptake of siderophores, haem, and vitamin B_{12}." Microbiology. 1998: **144**, 2759-2769.

Hamza, Iqbal, Richard Hassett, Mark R. O'Brian. "Identification of a Functional *fur* gene in *Bradyrhizobium japonicum*." Journal of Bacteriology. Sept. 1999: 5843-5846.

Heinrichs, Jon H., LaVette E. Gatlin, Charles Kunsch, Gil H. Choi, Mark S. Hanson. "Identification and Characterization of SirA, an Iron-Regulated Protein from *Staphylococcus aureus*." Journal of Bacteriology. Mar. 1999: 1436-1443.

Hirst, P.H., S.J. Mills, I.D. Spiers, D.R. Poyner, S. Freeman, B.V.L. Potter, A.W. Smith. "Inositol Polyphosphate-mediated Iron Transport in *Pseudomonas aeruginosa*." Journal of Applied Microbiology. 1999: **86**, 537-543.

Lewis, L.A., M.H. Sung, M. Gipson, K. Hartman, D.W. Dyer. "Transport of Intact Porphyrin by HpuAB, the Hemoglobin-Haptoglobin Utilization System of *Neisseria meningitidis*." Journal of Bacteriology. Nov. 1998: 6043-6046.

Liles, Mark R., Tracy Aber Scheel, Nicholas P. Cianciotto. "Discovery of a Nonclassical Siderophore, Legiobactin, Produced by Strains of *Legionella pneumophila*." Journal of Bacteriology. Feb. 2000: 749-755.

Nicholson, Mary Lou and Bernard Beall. "Disruption of *tonB* in *Bordetella bronchiseptica* and *Bordetella pertussis* Prevents Utilization of Ferric Siderophores, Haemin, and Haemoglobin as Iron Sources." Microbiology. 1999: **145**, 2453-2461.

Schubert, S., D. Fischer, J. Heesemann. "Ferric Enterochelin Transport in *Yersinia enterocolitica*: Molecular and Evolutionary Aspects." Journal of Bacteriology. Oct. 1999: 6387-6394.

Weinberg, Eugene, ed. Microorganisms and Minerals. New York: Marcel Dekker, Inc., 1977.

Zhu, Wenming, Desiree J. Hunt, Anthony R. Richardson, Igor Stojiljkovic. "Use of Heme Compounds as Iron Sources by Pathogenic Neisseriae Requires the Product of the *hemO* gene." Journal of Bacteriology. Jan. 2000: 439-446.