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J. Clark, *et al.*

Heme Catabolism in the Causative Agent of Anthrax

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Systemic anthrax presents as a dramatic and overwhelming bacterial infection (WHO, 2008). Always serious, often fatal even with the best modern medicine has to offer, it's onset is a quiet incubation period when symptoms remain mild as compared with many other acute bacterial infections. This period is a time of deadly expansion. After *Bacillus anthracis* spores gain entry into the body (inhalation of aerosolized spores is one well-characterized threat), they travel to the reginal lymph nodes and germinate (Dixon *et al.,* 1999). Germination is followed rapidly by initial replication with expression of virulence factors, escape into the blood and, then, hematogenous spread throughout the victim – with intensive replication at each stage. Systemic anthrax often becomes a massive infection spreading to all sites in the body. Germination of just a few spores, within short order, results in bacilli numbers of 10^8 organisms per cc of blood, or even higher! Death often occurs within days of initial symptoms.

The crucial actions and contributions of the *B. anthracis* exotoxins, capsule and other virulence systems to pathologies have been extensively investigated and insights exploited as the basis for medical countermeasures (Dixon *et al.,* 1999; WHO 2008). However, a deep examination of the various nutrient acquisition systems required by this microbe – specifically, those that satisfy the large metabolic requirements that accompany its explosive growth in

numbers - are now being categorized and evaluated. Key to meeting one of these crucial nutrient requirements are the multi-abilities of *B. anthracis* to acquire iron.

Mining iron from the host is an absolute necessity for the vast majority of bacterial pathogens (Hood and Skaar, 2012; Contreras *et al.,* 2014; Wandersman and Delepelair, 2014), and for this - as with many other bacterial pathogens - *B. anthracis* is well known to make use of an iron-chelating siderophore. The *B. anthracis* siderophore contributing to systemic anthrax is named petrobactin, a type of "stealth" siderophore (Cendrowski *et al.,* 2004; Abergel et al., 2006). Siderophores are secreted, small-molecule scavengers of free iron and also have the ability to remove bound iron from various host proteins/factors. After binding "host iron," they return to deliver the metal for the bacteria's own uses. Mutational studies showed that the petrobactin system is absolutely essential for *B. anthracis* growth under low-iron conditions, *in vitro*, and for systemic anthrax infections in mice (Cendrowski *et al.,* 2004). Bacillibactin, another *B. anthracis* siderophore, so far appears not to play an active role during anthrax (Cendrowski *et al*., 2004). However, new findings in the accompanying article by Justin Clark *et al.* (cite: MMI-2018-17212.R2), show that petrobactin cannot meet all the iron needs of *B. anthracis*.

Heme, a porphyrn ring with a central iron molecule, is abundant in red blood cells in the context of hemoglobin, and in other host cells (in various forms). It is essential for oxygen exchange and numerous other cellular processes. To a bacterial pathogen, heme also represents a potentially rich source of iron; one that may not be siderophore-accessible (Wandersman and Delepelair, 2014). Earlier work showed *B. anthracis* IsdG, a cytoplasmic heme monooxygenase, bound and degraded heme (Skaar *et al.,* 2006). In that study, it also was observed that IsdG mutants were not attenuated in spore-challenged mice, confounding a potential role of heme as a contributing iron source. With those observations, it would seem easy enough simply to "assign" the duties of iron collection to petrobactin, and to relegate the IsdG heme monooxygenase to some other "unknown" job.

But was it possible that, despite the lack of attenuation seen with the IsdG mutant, heme monooxygenase systems do serve in acquiring iron during anthrax, alongside of petrobactin?

The pathogen's explosive growth likely necessitates a need for lots of iron. Multiple ways of obtaining that iron potentially would be of great advantage. Also, other pathogenic bacteria use heme monooxygenases for acquiring iron and IsdG is fully capable of catabolizing heme *in vitro* (Owens *et al*., 2013; Haley *et al.* 2011; Wandersman and Delepelair, 2014; Skaar *et al.,* 2006). To one group of investigators, the non-attenuation of the IsdG mutant suggested something challenging: redundancies within redundant systems. Specifically, that *B. anthracis* encoded multiple types of pathways for iron acquisition (heme-based *and* siderophore-based), that *more than one* heme-based system (*i.e.,* more than IsdG alone) promotes heme-utilization and that heme sources *are required* to meet the total iron required for systemic anthrax infections.

All three hypotheses proved correct! In the accompanying study, Clark *et al*. identify and characterize not just one, but two additional heme-binding and -degrading hemeoxygenases, named HmoA and HmoB (see the model figure in their article). Their work indicates that, including IsdG, *B. anthracis* maintains at least three known enzymes with potential to access iron from host heme. To determine essentiality of these enzymes during infection, combinations of deletion mutants were generated and mice challenged with spores. As anticipated, all single gene deletion mutants were found to be just as virulent as wild-type *B. anthracis*, but isogenic strains missing a combination of *both* IsdG *and* HmoA were severely attenuated, thus underscoring the overlapping contribution of these two critical, heme-centric systems. Petrobactin synthesis genes were not altered in their studies indicating that the siderophore, alone, could not supply the pathogen with sufficient iron. Mutant constructs deficient in HmoB did not impact overall disease severity as significantly as those missing IsdG + HmoA, but did increase bacterial clearance from lungs, suggesting more of a niche (and/or other) role for this particular heme monooxygenase.

Clark *et al.'s* discoveries now show conclusively that *B. anthracis* has multiple and redundant systems to catabolize heme and thus realize greater potential sources of host iron. Examples of heme exploitation is found in *M. tuberculosis* and *S. aureus* and several other bacterial pathogens (Owens *et al*., 2013; Haley *et al.* 2011; Wandersman and Delepelair, 2014). The functional redundancy of IsdG with HmoA, along with the severe attenuation observed for the IsdG/HmoA double mutant, suggest two important things: that ability to catabolize heme is

absolutely essential to successful systemic anthrax infections and that the petrobactin siderophore alone (although also essential for infection) cannot meet the *B. anthracis* iron nutrient requirements *in vivo*. Both the heme and the siderophore systems are required by this pathogen.

The specific biological requirements for *B. anthracis* maintaining multiple, redundant iron acquisition systems remains a matter of speculation. Why so many ways to secure iron? Centrally implicated (if often vaguely interpreted) is the notion of "great need." Simply put, bacteria need the iron. The extreme growth patterns seen during systemic anthrax suggested iron acquisition is a very high priority. While host iron sources seem relatively abundant, mammals maintain multiple types of robust sequestration and regulatory systems in place – in part, to mitigate its toxicities. The metal is there, but safely bound and tricky to ferret out. Specific differences in how the host secures its own iron may require a variety of extraction methods by the pathogen. As a possible example, although a disease that progresses rapidly, systemic anthrax occurs in stages. There is an early intracellular stage (macrophages), followed by localized lymphatic growth and spread in the blood and throughout the various tissues. Multiple extraction methods may facilitate efficient iron acquisition in the variety of micro-environments encountered. Similar thinking might be applied at the species tropism level. Anthrax is panzootic, most mammalian species (and a few non-mammalian) are susceptible. Multiple mining methods may allow for *B. anthracis* to acquire its iron from different hosts, thus meeting challenges of fine-level variations in iron sequestration (and immune) systems that are species-specific. Finally, storage of some iron is, potentially, an advantage to the bacteria. Not all the iron obtained is immediately put to work. Storing any extra iron for use on a rainy day seems prudent but, as with the host, excess un-bound iron is toxic to the bacterium. So too is excess heme. Potential "safe storage" and/or detox functions have been hypothesized for the bacillbactin siderophore and, now, by Clark *et al.*, also for the heme monooxygenases. Deeper understanding of the various metabolic coordination and transcriptional regulatory controls of *B. anthracis* iron metabolism should reveal important details on how, and perhaps why, multiple redundancies in iron metabolism are so essential in anthrax.

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