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Salmochelin, the long-overlooked catecholate siderophore of *Salmonella*

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Abstract Salmochelin is a C-glucosylated enterobactin produced by *Salmonella* species, uropathogenic and avian pathogenic *Escherichia coli* strains, and certain *Klebsiella* strains. It was the first glucosylated siderophore described. The glucosylation has been interpreted as a bacterial evasion mechanism against the mammalian catecholate siderophore-binding protein siderocalin (NGAL-lipocalin). The synthesis, excretion, and uptake of salmochelin requires five genes, *iroBCDEN*, and also the enterobactin biosynthesis and utilization system. Some salmochelin-producing strains also secrete microcins, which possess a C-terminal, linear glucosyl-enterobactin moiety. These microcins recognize the catecholate siderophore receptors IroN, Cir, Fiu, and FepA, and may inhibit the growth of competitors for catecholate siderophores.

Keywords Salmochelin · Iron transport · Siderocalin · *Salmonella* · C-glucosylation · Microcin E492

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Introduction

Around 1940, after many vitamins had been identified, Howard Schneider und Leslie Webster at the Rockefeller Institute in New York began to search for compounds in the daily diet that protect against infections. Their model system was mice infected with *Salmonella enterica* serovar Typhimurium. For one group of mice, they developed a synthetic, well-defined diet containing all known vitamins. A second group of mice received a "natural" diet with high nutritive value. Mice on the synthetic diet were more susceptible to infection than mice on the natural diet. Attempts to extract the responsible protective factor from different fractions of the natural diet (e.g., from grain) proved to be extremely difficult and irreproducible. Finally, a substance called pacifarin ("peace bringing") was extracted from one component of the natural diet, commercial dried hen egg white (Schneider 1967).

To obtain pure pacifarin, researchers started to make their own dried hen egg white, but the home-made dried egg white surprisingly did not contain the substance that protected the mice from *Salmonella* infection. Backtracking the commercial source revealed that the egg white was not dried immediately, but rather allowed to go through a "fermentation" period to stop the dried egg white from turning brown during its shelf-life; the sugars responsible for the age browning are degraded during the microbial fermentation, and the egg white remains stably white. One of the bacteria from the egg

white fermentation, *Aerobacter* sp., was cultivated, and pacifarin was isolated; this was only possible when the strain was grown under iron-limiting conditions (Wawszkiewicz and Schneider 1975). Egg white is a very iron-poor substrate for bacteria because it contains ovotransferrin, which avidly binds iron in a form not accessible for most bacteria. The bacteria growing on egg white, therefore, are expected to produce siderophores (iron chelators) to overcome the iron limitation.

Analysis of pacifarin indicated that catechol and serine are constituents. These compounds had been found earlier in the iron-complexing siderophore enterobactin of *Escherichia coli*. Enterobactin has pacifarin-like properties and is produced when *E. coli* is grown in iron-poor surroundings. Therefore, enterobactin was used in the studies on diet components that protect against infection, and was fed to mice on a synthetic diet. More of these mice survived the *Salmonella* infection than those on the synthetic diet without enterobactin. This finding diminished the interest in the originally isolated pacifarin, even though it was known that the hydrophilic pacifarin isolated from *Aerobacter* must differ chemically from the more hydrophobic enterobactin. After 300,000 mice were sacrificed for the isolation of pacifarin, the institute studying this compound was closed (Wawszkiewicz 1975) and the rest of the pacifarin was given to chemists for structure determination. In 1985, the structure of pacifarinic acid was published in a thesis (Fu 1985), and it was claimed to be part of pacifarin. Still there was no further interest in pacifarin for many years.

The Iro operon and salmochelin

In 1995, an operon of iron-regulated genes, *iroBCDE* and *iroN* (Fig. 1), was identified in *Salmonella* (Bäumler et al. 1996). IroN is highly similar to the enterobactin receptor FepA, IroD is similar to the enterobactin esterase Fes, and IroB is highly similar to glycosyltransferases; and indeed a glucose-containing enterobactin produced by *Salmonella* was identified and called salmochelin (Hantke et al. 2003). Salmochelin S4 is a twofold C-glucosylated enterobactin (Fig. 2; Bister et al. 2004). A literature search revealed that the salmochelin monomer

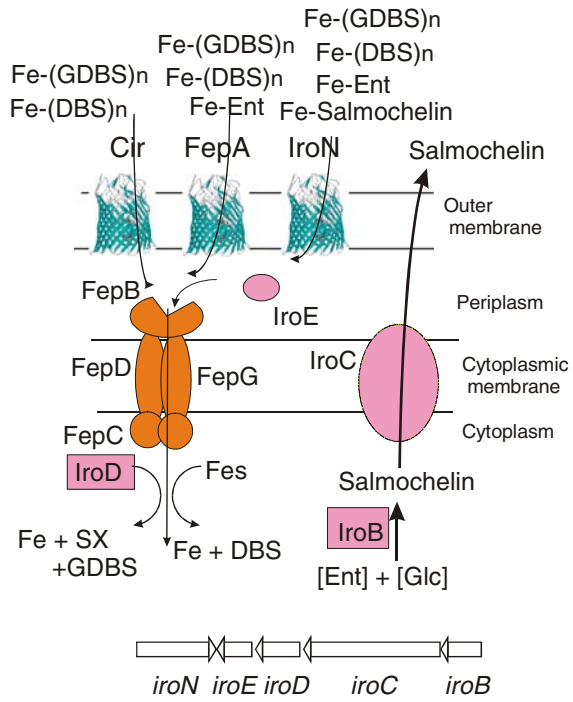

Fig. 1 A model of the interplay of the salmochelin biosynthesis and utilization systems with the enterobactin systems. Enterobactin (Ent) is glucosylated (Glc) by IroB and secreted with the help of IroC. Ferric iron complexed in the medium by cyclic salmochelin S4 (see Fig. 2) is taken up via IroN. The cyclic tri-ester might be linearized in the periplasm by IroE. The major uptake pathway into the cytoplasm is via the FepBCDG ABC transporter. In the cytoplasm, the salmochelins may be split further by IroD, and the degradation products [glucosylated 2,3-dihydroxybenzoylserine monomers (=SX, pacifarin acid), dimers, and trimers $(GDBS)_n$] are exported and might be reused as siderophores

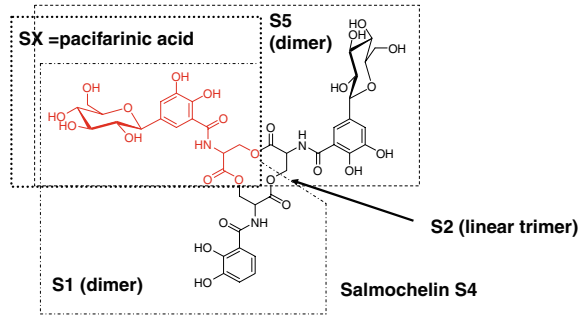

Fig. 2 Structure of salmochelin S4 and its degradation products

C5-β-glucosyl-2,3-dihydroxybenzoyl serine was actually the earlier described pacifarinic acid (Fu 1985), i.e., that salmochelin S4 is pacifarin. Therefore, not

only enterobactin but possibly also salmochelin may stimulate the innate immune system of the mice. These experiments have not been repeated with purified salmochelin, and the protection mechanism has not yet been elucidated.

In this context, it is interesting to note that the mammalian protein NGAL-lipocalin (siderocalin) binds enterobactin (Goetz et al. 2002). NGAL-lipocalin has been detected in granula of neutrophiles (Kjeldsen et al. 1993), is produced by epithelial cells during inflammation, and is part of the mammalian innate immune response (Flo et al. 2004). It protects mice against *E. coli* infections. In serum, iron is strongly bound by transferrin and is therefore not available for bacteria unless they are able to take the iron away from transferrin. This is possible with siderophores under certain conditions. Binding of the catecholate bacterial siderophores by NGAL-lipocalin is a counterstrike of the mammalian host against the bacteria fishing for iron.

Commensal and parasitic bacteria have additional means of scavenging iron. Besides producing non-catecholate siderophores, certain bacteria are able to glucosylate enterobactin to form salmochelin. Salmochelin, with its two glucosyl residues, is not bound by NGAL-lipocalin, and a salmochelin-producing *E. coli* strain is not restricted by NGAL-lipocalin in a mouse infection model (Fischbach et al. 2006). Similarly, the growth inhibition of the standard laboratory strain *E. coli* K-12 by NGAL-lipocalin is abolished when the strain is transformed with a plasmid enabling the production of salmochelin (Valdebenito et al. 2007).

The salmochelin receptor IroN

What is known about the synthesis and transport of the first glucosylated siderophore described? Many functions of the *iroN* and *iroBCDE* genes could be surmised by comparing the deduced protein sequences with sequences in the database. The predicted IroN protein shows a high level of identity (52%) to FepA, the TonB-dependent outer membrane receptor for enterobactin. Transport experiments and growth promotion tests revealed that IroN is the outer membrane receptor for salmochelin (Fig. 1; Hantke et al. 2003) with a broad specificity also for other siderophores (Rabsch et al. 2003). It has recently been shown that IroN also has an accessory function in invasion (Feldmann et al. 2007).

The glucosyltransferase IroB

The predicted IroB protein shows 33% identity to the SnogD protein, which is a glycosyltransferase involved in the biosynthesis of the anthracyclin antibiotic nogalamycin produced by *Streptomyces nogalater* (Torkkell et al. 2000). It was shown in vivo (Bister et al. 2004) and in vitro (Fischbach et al. 2005) that IroB is the enzyme that C-glucosylates enterobactin. Attempts to identify the natural nucleoside diphosphate glucose substrate of IroB were unsuccessful. Mutants in *galU, glgC, rfbF,* or *rmlA* or *rffH*, which encode enzymes that activate glucose with UTP, ATP, CTP, or dTTP, respectively, were still able to produce salmochelin (unpublished observations), which might indicate that glucose activated with various nucleotide triphosphates can be used as a substrate. A BLAST search for enzymes more closely related to IroB indicates that this protein is only found in the group of enterobacteria that includes *Salmonella*, *Escherichia, Shigella*, and *Klebsiella*, and is not found in other Gram-negative bacteria. These *iroB*-like genes are always found in a cluster with *iroCDEN* genes.

Genes encoding proteins with high levels of identity to IroB are also found in certain gene clusters involved in the colicin-V-related microcin biosynthesis; this will be discussed below in more detail. One ORF encoding a putative protein with a high level of similarity to IroB is found in the Gram-positive bacterium *Brevibacterium linens* BL2.

The MDR protein IroC

The IroC protein has considerable similarities to mammalian ATP-driven MDR (multiple drug resistance) exporter proteins. Therefore, in the first analysis of the *iro* genes, it was assumed that IroC facilitates export of glucosylated salmochelin (Hantke et al. 2003). However, linear salmochelin S2 stimulated the growth of an *E. coli fepB* mutant complemented with an *iroBCDEN* plasmid; since FepB is the binding protein required for Fe^{3+}-enterobactin transport, (Zhu et al. 2005) concluded that IroC facilitates

salmochelin uptake. Recently it has been shown that IroC in *S. enterica* exports salmochelin and that mutants in the membrane component FepD of the enterobactin ABC transporter are unable to use salmochelin and enterobactin (Crouch et al. 2008).

The esterases IroD and IroE

The IroD protein was assumed to have an esterase function owing to its similarity to the enterobactin esterase Fes. Indeed, the purified protein cleaves cyclic salmochelin to the linear trimer, dimer, and monomer (Zhu et al. 2005). The iron-containing siderophore is only cleaved to appreciable amounts when ferrozine, a ferrous–iron-complexing chelator, is added (Zhu et al. 2005). The major function of IroD in the cell is thought to be the cleavage of ferric iron salmochelin to lower the affinity of iron to the chelator (Lin et al. 2005). Surprisingly, the enzyme was apparently inactive with the substrate iron-salmochelin; however, this can be explained by a rapid product inhibition of the enzyme reaction. This product inhibition avoids accumulation of free iron in the cell when there is no iron chaperone present to take care of this toxic metal.

The IroE protein is localized in the periplasm. It degrades cyclic salmochelin and cyclic ferric salmochelin to the linear form (Zhu et al. 2005; Lin et al. 2005). In contrast to IroD, this esterase does not cleave the linear salmochelin further to monomers and dimers. The structure of the dimer salmochelin S1 found in the supernatant shows that the esterases cleave preferentially at the carboxyl group of the non-glucosylated 2,3-dihydroxybenzoyl-serine residue. Interestingly, an S1 isomer with the glucosyl residue attached to the 2,3-dihydroxybenzoyl-serine with a free carboxyl group was found in the supernatant of a microcin-M/H47-producing strain. This isomer has a slightly different HPLC retention time than S1.

PfeE (PA2690), a protein from *Pseudomonas aeruginosa* with 34% identity to IroE, is encoded downstream of the gene encoding the enterobactin receptor PfeA. PfeE also cleaves cyclic salmochelin S4 to linear salmochelin S2. *P. aeruginosa* utilizes enterobactin as a xenosiderophore, and since there are no homologues to the FepBCDE ABC transporter, cleavage of the cyclic catecholate siderophores lowers the affinity of the chelator for ferric iron. This might allow iron utilization through iron reduction and ferrous iron transport. Similar esterases are predicted in the periplasm of other Gram-negative bacteria based on genome sequences.

The relationship of the salmochelin system to the microcin E492, H47, and M systems

Interestingly, genes encoding IroB- and IroD-like proteins are also found in the biosynthesis gene clusters of the microcins E492, H47, and M (Patzer et al. 2003).The best-characterized microcin of this group is microcin E492, which is a peptide of 84 amino acids with the C-terminal serine modified with linear C-glucosyl enterobactin (Thomas et al. 2004; Mercado et al. 2008; Fig. 3). It is interesting to note that in this case enterobactin is only glucosylated once—in contrast to salmochelin, which is glucosylated twice. From the high sequence similarities, it is assumed that microcins H47 and M are similarly modified (Patzer et al. 2003). Considering the modification of these microcins, it is not surprising that their receptors are the catecholate siderophore receptors Cir, Fiu, IroN, and FepA (Patzer et al. 2003). In many cases, these microcin-producing strains are also able to produce salmochelin. Therefore, one may speculate that the microcins produced under iron-limiting conditions defend the salmochelin utilization system against competitors for this iron source.

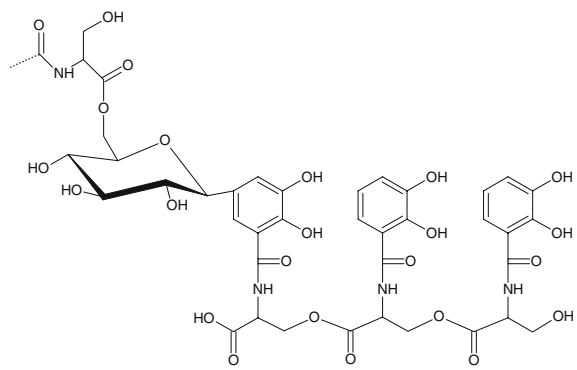

Fig. 3 C-terminal modification of microcin E492. The C-terminal Ser84 is linked by an ester bond to the sugar of a linear C-glucosyl-enterobactin (Thomas et al. 2004)

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