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Characterization of Metal Transport by the Streptococcus sanguinis Endocarditis Virulence Factor SsaB

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Streptococcus sanguinis and Infective Endocarditis

- *Streptococcus sanguinis,* present in the biofilm colonizing human tooth surfaces, may be beneficial in the oral cavity, though it also serves as a causative agent of an extra-oral disease, infective endocarditis (Belda-Ferre *et al.*, 2012; Bor *et al.*, 2013).
- Infective endocarditis, infection and inflammation of the heart valves or endocardium, diminishes proper functioning capabilities of the heart. Complications include congestive heart failure, aneurysm, and stroke (Bashore *et al.*, 2006).
- *S. sanguinis* enters the bloodstream through lacerations of the oral cavity and may attach to pre-existing vegetations composed of platelets and fibrin formed previously in response to cardiac injury.
- Dental procedures, routine oral hygiene maintenance, and chewing can lead to this transient bacteremia (Wilson *et al.*, 2007).

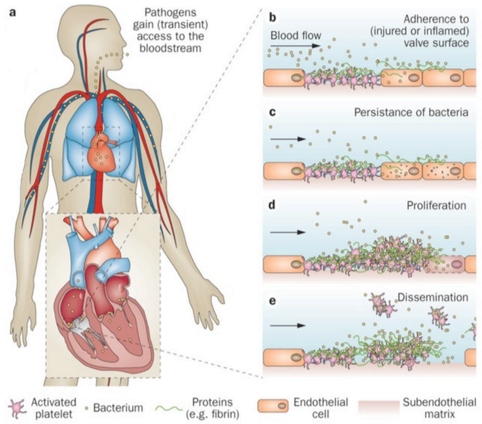


Figure 1. Mechanism of bacterial adherence to heart valve or endocardium following transient entry into bloodstream through oral cavity laceration (Werdan *et al.*, 2013).

SsaB is Critical for Virulence

- Previous mutagenesis of lipoprotein genes from *S. sanguinis* wild-type strain SK36 identified the SsaB gene as necessary for virulence in a rabbit model of endocarditis (Das *et al.*, 2009).
- SsaB, orthologous to previously characterized Lipoprotein Receptor Antigen I proteins important to the virulence of related streptococcal species, was assigned putative function as the substrate-binding lipoprotein of an ATP-binding cassette (ABC) transport system (Jenkinson, 1994; Claverys, 2001).
- Based on homology, the SsaABC system was hypothesized to transport manganese and, potentially, other divalent metal cations, particularly iron (Janulczyk *et al.*, 2003; McAllister *et al.*, 2004).
- Accumulated manganese was expected to contribute to virulence by acting as cofactor for the manganese-dependent superoxide dismutase, SodA, providing resistance to oxidative stress (Johnston *et al.*, 2004).

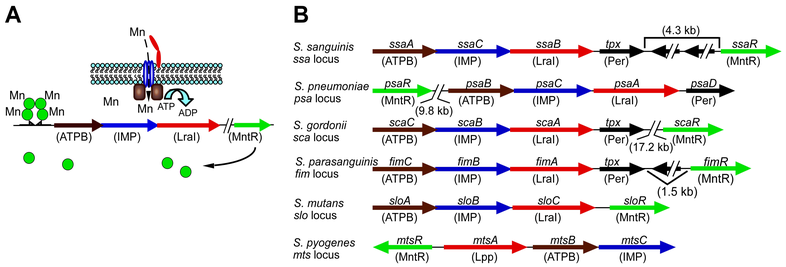


Figure 2. LraI operon organization and predicted gene functions. (A) Model of a generic LraI operon, including repression mediated by Mn^{2+}-bound MntR. (B) Comparison of LraI operons from representative streptococcal species. Genes depicted in the same color are orthologous. ATPB: ATP-binding protein; IMP: integral membrane permease; LraI: substrate-binding lipoprotein; Per: thiol peroxidase; MntR: MntR-family regulator. Sequences taken from accession numbers NC_009009, AE005672, NC_009785, NC_017905, AF232688, and NC_004070, in the order depicted.

Determination of Metal Content Using Inductively Coupled Plasma Optical Emission Spectrometry

- For experiments involving growth in brain-heart infusion (BHI) broth (Difco), cells were cultured overnight in 80% BHI/20% pooled rabbit serum in 6% O_2. Three ml of each culture was then added to 36 ml BHI broth that had been pre-incubated under the same conditions. Incubation was continued at 37°C for 5 hours.
- For growth in all-purpose tween (APT) broth (Difco), cells were cultured anaerobically overnight in APT broth, then diluted as above in pre-warmed APT and incubated at 37°C for 5 hours. *E. coli* cells were incubated with shaking (225 rpm), while the other strains were incubated statically in tightly-capped tubes containing anaerobically pre-incubated APT.
- Cells were harvested by centrifugation at 4°C for 10 min at 3,743 x *g* and washed twice in 10 ml cold phosphate-buffered saline (PBS) that had been pre-treated with Chelex-100 Resin (BioRad) and supplemented with 1 mM EDTA. Cell pellets were suspended in 1 ml concentrated nitric acid (HNO_3) (Fisher Scientific) and incubated at 95°C for 1 hour; 900 ml of this was added to 9.1 ml dH_2O.
- Metal composition was determined using an MPX Vista inductively coupled plasma-optical emission spectrometer (Varian, Inc.). Concentrations were determined by comparison with a standard curve created with a 10 µg ml^{-1} multi-element standard (CMS-5; Inorganic Ventures) diluted in dH_2O and then two-fold in 1% HNO_3 to generate dilutions ranging from 5.12 to 0.02 µg ml^{-1}.
- To avoid contamination with metals, trace-metal grade HNO_3 was used, all dH_2O was treated with Chelex prior to use, and all glass and plastic vessels used for metal analysis were soaked overnight in 1 M HNO_3 prior to use.

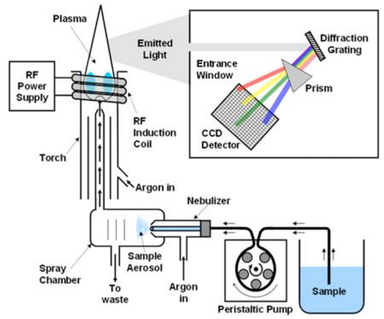


Figure 3. Basic Design of the ICP-OES Instrument (Concordia Analytical Instrument Laboratory, 2014).

SsaB Mediates Manganese and Iron Accumulation

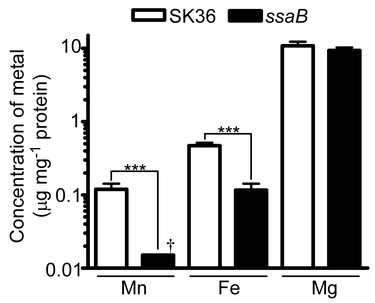
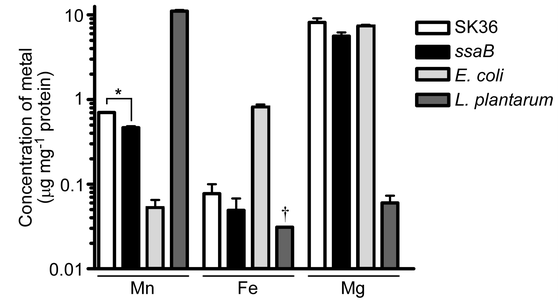


Figure 4. Analysis of metal content by ICP-OES SK36 and *ssaB* mutant cells grown in BHI broth. Means and standard deviations from n=3 independent experiments are shown. † represents value below the limit of detection (0.016 µg mg^{-1} cellular protein). The means and standard deviations from n=3 independent experiments are shown.

- The *ssaB* mutant reproducibly accumulated lower concentrations of both manganese and iron.
- No significant differences were observed in accumulated concentrations of magnesium, or any of 14 additional metals for which there were standards, or in signal intensities for 60 other elements analyzed for which there were no standards.
- This data suggested that SsaB binds both manganese and iron to facilitate transport.
- When grown in BHI broth, both strains accumulated more iron than manganese.
- Next, SK36 and the ssaB mutant were analyzed following growth in APT broth, rich in both metals, to assess whether this was due entirely to the higher concentration of iron relative to manganese in BHI broth.

Analysis of Metal Content by ICP-OES in Cells Grown in APT Broth

Figure 5. Analysis of metal content by ICP-OES in cells grown in APT broth. Strains analyzed were SK36, *ssaB* mutant, *Escherichia coli* (DH10B), and *Lactobacillus plantarum* (14917). *E. coli* served as a control for a bacterium known to accumulate more Fe than Mn while *L. plantarum* is known to accumulate more Mn than Fe. † represents value below the limit of detection (0.031 µg mg^{-1} cellular protein). The means and standard deviations from n=3 independent experiments are shown.



- Higher accumulation of manganese than iron in APT broth compared to BHI broth for both SK36 and the *ssaB* mutant suggested that manganese is accumulated through both SsaB-dependent and SsaB-independent mechanisms.
- Relative cellular abundance of iron and manganese in *S. sanguinis* varies dramatically depending on relative abundance in the growth medium, highlighting the importance of using physiologically relevant media in future studies.
- This data also implies that *S. sanguinis* is flexible in its metal requirements and is rather efficient in sequestering iron, which would otherwise react with cellular hydrogen peroxide to produce DNA-damaging hydroxyl radicals via the Fenton reaction.

Table 1. ICP-OES-determined concentrations (µg mg^{-1} protein) of the 14 additional metals for which there were standards in cells of SK36, *Escherichia coli* (DH10B), and *Lactobacillus plantarum* (14917). Negative values were below the limit of detection for the element analyzed. The means from n=3 independent experiments are shown.

	SK36	E. coli	L. plantarum
Silver (Ag)	0.021	0.008	0.006
Aluminum (Al)	-0.017	0.013	-0.001
Calcium (Ca)	-3.355	-1.203	-1.207
Cobalt (Co)	-0.025	-0.010	-0.010
Chromium (Cr)	-0.018	-0.003	-0.004
Cesium (Cs)	-0.727	-0.367	-0.115
Copper (Cu)	0.033	0.029	0.027
Potassium (K)	13.160	51.382	6.478
Lithium (Li)	0.002	0.002	0.001
Sodium (Na)	-136.738	0.001	-64.807
Nickel (Ni)	-0.066	-0.001	-0.018
Rubidium (Rb)	-0.012	0.003	-0.003
Strontium (Sr)	0.002	0.001	-0.001
Zinc (Zn)	-1.045	-0.364	-0.277

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