# Human Hemoglobin Polymorphisms Affect Recognition by *S. aureus* Receptor IsdB

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BRIEF. Bacterial adenylate cyclase two-hybrid assay shows that hemoglobin variants are differentially bound by *S. aureus* receptor IsdB.

ABSTRACT. Iron is an essential nutrient for the survival of *Staphylococcus aureus*. During infection, the body sequesters iron as a method of nutritional immunity. However, *S. aureus* can utilize the protein IsdB to bind hemoglobin, from which iron can be extracted and metabolized. In the human population, there are over a thousand characterized polymorphisms of hemoglobin. It is unknown whether these natural variations are bound differently by IsdB. Eight naturally occurring mutations were selected for their proximity to sites previously identified to be recognized by another *S. aureus* surface protein, IsdH. The levels of interaction between IsdB and these hemoglobin variants were measured using the bacterial adenylate cyclase two-hybrid system, which was optimized for this particular study. Experimentation identified five hemoglobin variants (αD6Y, αK7E, αK11Q, αD75Y, and αN78K) that demonstrated significantly lower recognition by IsdB. Of these variants, the first four are very rare. The αN78K variant is relatively more common, found in many populations of African origin. These results demonstrate that natural hemoglobin variants can be bound differently by *S. aureus* receptor IsdB. Hemoglobin genotype, then, is a potentially important factor to consider during treatment of infections. The possible need for individualized therapies is thus suggested.

## INTRODUCTION.

*Staphylococcus aureus* is a bacterial species that colonizes over 30% of the American population [1]. Upon rupture of defensive skin or mucosal layers, *S. aureus* can invade the body [2]. In this manner, *S. aureus* is responsible for a variety of diseases ranging from mild food poisoning to deadly infection [3]. Through such diseases, *S. aureus* caused 300,000 hospitalizations and 40,000 deaths in 2005 [4]. It is evident that *S. aureus* is a potent pathogen responsible for widespread mortality.

During infection, one of the body's primary defenses is to withhold iron, a vital nutrient, from the invading microorganism [3]. Free iron is isolated in proteins within cells, where it is difficult to access [3]. Nearly 75% of the body's iron is stored in hemoglobin, the oxygen-transport protein, which is further secluded within red blood cells [5]. *S. aureus* is exceptionally infectious partially because it can withdraw iron from hemoglobin [3]. It does this using the iron-regulated surface determinant system (Isd), an arrangement of surface proteins that collectively bind hemoglobin and acquire iron [6].

IsdB has been established as the primary hemoglobin receptor of *S. aureus* [7]. The ability of IsdB to bind hemoglobin thus largely determines the efficiency of iron-acquisition and subsequently of infection. Because variations in human hemoglobin may affect IsdB's efficiency in binding hemoglobin, it is important to understand the specificity of IsdB to hemoglobin variants.

Diversity of hemoglobin arises from natural mutations, or polymorphisms, in genomic DNA. Different polymorphisms have resulted in the characterization of over a thousand human hemoglobin variants globally [8]. Here, we present a study on the response of IsdB to various natural human hemoglobin variants.

A previous study identified hemoglobin sites near αK7, αT8, αK11, and αD74 ("α" designates location on the hemoglobin α-chain) as critical for binding by near iron transporter domain 1 of another *S. aureus* cell surface receptor, IsdH (Figure 1) [9].

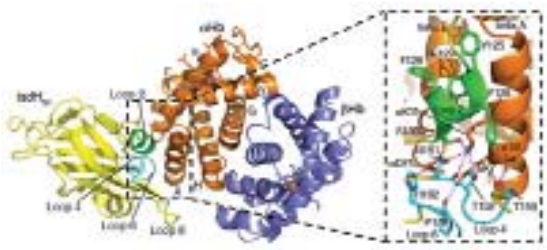

**Figure 1.** X-ray crystallography model of a hemoglobin dimer (α-chain in orange, β-chain in blue) bound by IsdH near iron transporter domain 1 (yellow) [9]. Enlargement displays sites critical to the interaction between hemoglobin and IsdH.

Because IsdB is similar to IsdH in function and sequence [9], it was hypothesized that hemoglobin sites near αK7, αT8, αK11, and αD74 would also be important to hemoglobin's interaction with IsdB. Representative hemoglobin polymorphisms near α-chain sites αK7, αT8, αK11, and αD74 and on randomly selected β-chain sites were examined in this study. It was theorized that the α-chain polymorphisms would decrease levels of interaction with IsdB and that the β-chain polymorphisms would cause little effect.

To test the above hypothesis, the bacterial adenylate cyclase two-hybrid (BACTH) system was applied. In this system, hemoglobin and IsdB are tagged with either the T18 or T25 fragment of adenylate cyclase [10]. If capable of interacting, hemoglobin and IsdB bring the two fragments together [10]. The resulting active adenylate cyclase causes *Escherichia coli* to digest a reporter substance that changes color when broken down [10]. A measure of color change is therefore a measurement of protein interaction [10]. Using this system, the levels of interaction between the aforementioned hemoglobin variants and IsdB were measured.

## MATERIALS AND METHODS.

### *Gene Cloning.*

Plasmids were prepared using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA, Catalog #27104). The rHb gene was amplified from pHb0.0hug (10 ng/μL) by polymerase chain reaction (PCR). PCR conditions were: ①94°C for 1 minute, ②30 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 68°C for 1.5 minutes, ③68°C for 5 minutes, and ④4°C indefinitely. The rHb PCR product, pKNT25, and pKT25 were subjected to double restriction digest by BamHI and XmaI in NEBuffer 4 (New England BioLabs, Ipswich, MA, USA, Catalog #B7004S). Digestion products were isolated from agarose gel following electrophoresis using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA, Catalog #28704). Ligations of rHb with pKNT25 and with pKT25 were performed using T4 ligase. Ligation products were transformed into *Escherichia coli* strain DH5α. Colonies expressing kanamycin resistance (bestowed by pKNT25/pKT25 vectors) were selected. Plasmids were extracted from the selected transformants, visualized by gel electrophoresis, and sequenced.

### *Mutagenesis.*

Mutagenesis primers were phosphorylated using T7 polynucleotide kinase supplemented with ATP. Phosphorylated primers were used at 4 μM to induce point mutations in the pKNT25-rHb construct (10 ng/μL). High accuracy PfuTurbo DNA Polymerase (Agilent Technologies, Santa Clara, CA, USA, Catalog #600250) was used for mutagenesis PCRs. PCR conditions were identical to those described in section *Gene Cloning* except for those of the extension step, which was extended to 10 minutes at 68°C. PCR products were treated

with restriction enzyme DpnI to digest methylated template DNA. The samples were then ligated using T4 ligase and transformed into DH5α. Colonies expressing kanamycin resistance were selected. Plasmid constructs were visualized by gel electrophoresis and sequenced.

*Co-transformation.*

Combinations of plasmids pertaining to the bacterial adenylate cyclase two-hybrid (BACTH) assay were transformed into *E. coli* strain BTH10. Colonies expressing kanamycin and ampicillin resistance (bestowed by pKNT25/pKT25 and pUT18/pUT18C vectors respectively) were selected. The presence of plasmids in the selected transformants was examined by gel electrophoresis.

*Assay of β-galactosidase Activity.*

*E. coli* strain BTH101 harboring BACTH constructs were grown to stationary phase at 30°C in LB supplemented with 100 μg/mL ampicillin, 50 μg/mL kanamycin, and 1mM IPTG. Cultures were diluted 5 times with M63 medium (Amresco, Solon, OH, USA, Catalog #J910). $OD_{600}$ was recorded for the diluted suspensions and for a blank of diluted LB. Cells were permeabilized by adding 30 μL of toluene and 30 μL of 0.1% SDS per 2.5 mL of diluted suspension. Permeabilized cells were agitated, uncovered, at 37°C for 30 minutes to evaporate toluene. 0.25 mL of permeabilized cells were then added to 0.75 mL of PM2 assay buffer (70 mM $Na_2HPO_4$, 30 mM $NaH_2PO_4$, 1 mM $MgSO_4$, 0.2 mM $MnSO_4$, 100 mM β-mercaptoethanol, pH 7.0). A control reaction containing 1 mL of PM2 assay buffer was also included in the assay. To initiate the reactions, 0.25 mL of the substrate solution (4 mg/mL o-nitrophenol-β-galactoside in PM2 buffer without β-mercaptoethanol) was added. Reactions were carried out at 30°C, and after a color change was observed, the reactions were stopped by the addition of 0.5 mL of 1M $Na_2CO_3$. Reaction time in minutes was recorded. $OD_{420}$ was measured for the samples. To relate experimental measurements to enzymatic activity, the following equation was applied.

$$A = \frac{800 \times (OD_{420} - control\ OD_{420})}{reaction\ time \times 0.3 \times (OD_{600} - blank\ OD_{600})}$$

Value "A," the level of β-galactosidase activity, represented nmol of ONPG hydrolyzed per minute per mg of dry weight bacteria.

*Assay of β-galactosidase Activity Adapted for 96-well Plates [11].*

*E. coli* strain BTH101 harboring BACTH constructs was grown, supplemented with 0.3 mL of LB, 100 μg/mL ampicillin, 50 μg/mL kanamycin, and 1mM IPTG in a 96-well polypropylene block (2.2 mL volume per well) to stationary phase. Cells were diluted with 1.2 mL of M63 medium, and 175 μL of the diluted mixture were transferred to a microtiter plate. $OD_{595}$ was measured for each sample and for a blank (LB diluted with M63 medium). 180 μL of the diluted mixture were then permeabilized with 10 μL of chloroform and 10 μL of 0.05% SDS in a new 96-well polypropylene block. Permeabilized cells were diluted with 600 μL of M63 medium. 20 μL of the diluted permeabilized cells were mixed with 125 μL of assay mixture (0.8 mg/mL o-nitrophenol-β-galactoside in PM2 buffer, pre-equilibrated to 30°C for 5 minutes). A control well containing 145 μL of only assay mixture was also included in the assay. The reaction was carried out at 30°C for 45 minutes; 50 μL of 1M $Na_2CO_3$ was added to stop the reaction. $OD_{420}$ was measured for each sample. The following equation was used to relate the experimental measurements to enzymatic activity.

$$A = \frac{OD_{420} - control\ OD_{420}}{45 \times (OD_{595} - blank\ OD_{595})}$$

Value "A" in the equation represents relative β-galactosidase activity. Comparisons are valid only within the same experiment.

RESULTS.

*Vector Screen.*

rHb was inserted into vectors pKNT25 and pKT25. Constructs were confirmed by gel electrophoresis and DNA sequencing. Other constructs in the vector screen were readily available at the time of experimentation.

Combinations of relevant constructs were co-transformed and confirmed by gel electrophoresis (Figure S1).

The assay of β-galactosidase activity demonstrated discrepancies in levels of interaction reported by the examined vector combinations (Figure 2).

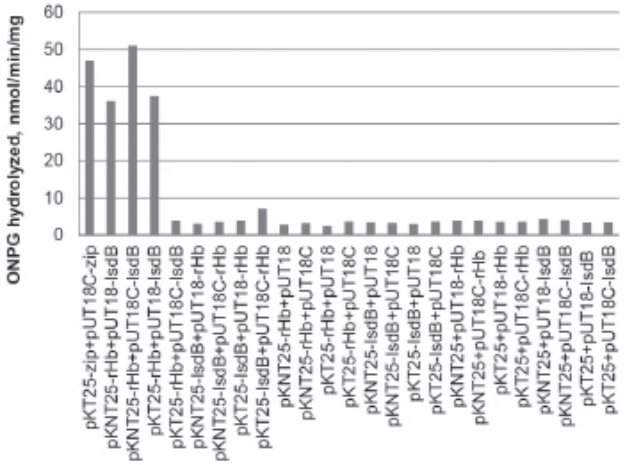


**Figure 2.** Vector screen via assay of β-galactosidase activity. The first sample from the left is a manufacturer-provided positive control containing two leucine zippers known to interact in the bacterial two-hybrid system. The next eight are experimental combinations, containing both rHb and IsdB. The remaining samples are negative controls, lacking either rHb or IsdB.

*Variant Screen.*

Ten hemoglobin variants (αS3F, αA5D, αD6Y, αK7E, αK11Q, αH72Y, αD75Y, αN78K, βV20E, and βV126A) were generated in construct pKNT25-rHb. Constructs were first confirmed via gel electrophoresis; plasmids of the correct size were sequenced to verify the presence of mutation and the integrity of T25.

pKNT25-variant constructs were co-transformed with pUT18C-IsdB. Co-transformants were confirmed by gel electrophoresis (Figure S2).

The assay of β-galactosidase activity adapted for 96-well plate demonstrated discrepancies in the levels of interaction between different hemoglobin variants and IsdB (Figure 3).

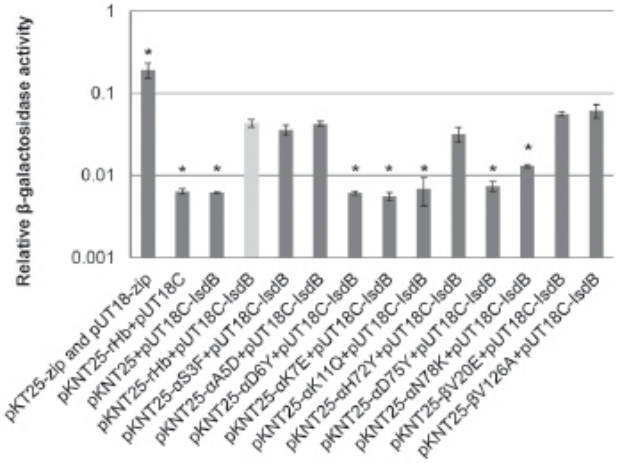


**Figure 3.** Variant screen via assay of β-galactosidase activity modified for 96-well plates. The first sample from the left is a manufacturer-provided positive control, which contains two leucine zippers known to interact in the bacterial two-hybrid system. The next two are negative controls, missing either rHb or IsdB. The rest are experimental combinations, containing both a hemoglobin variant and IsdB. Standard deviation is shown. Stars symbolize significant difference ($p<0.001$) from the fourth sample (colored light gray), which is the level of interaction between the predominant variant of rHb and IsdB.

DISCUSSION.

*Vector Screen.*

An initial screen was performed to determine the combinations of constructs best suited for reporting levels of interaction between hemoglobin and IsdB.

This vector screen revealed construct combinations of pKNT25-rHb with pUT18-IsdB or pUT18C-IsdB and of pKT25-rHb with pUT18-IsdB as optimal (Figure 2). Of the three positive combinations, pKNT25-rHb with pUT18C-IsdB produced the greatest signal. It was thus hypothesized to be able to display the greatest fluctuations in levels of interaction between IsdB and Hb variants.

The results of the initial vector screen indicated not only which combination of constructs was ideal for reporting the interaction between hemoglobin and IsdB, but they furthermore demonstrated the capability of the BACTH system. Previous research has characterized *in vivo* the interactions between proteins hemoglobin and IsdB [12]. The present project validated a complementary *in vitro* method of examining the same interactions.

*Variant Screen.*

Applying the findings of the vector screen, hemoglobin variants were tested for interaction with IsdB in the pKNT25-rHb/pUT18C-IsdB construct combination.

From the present data, it was observed that neither βV20E nor βV126A variants interacted with IsdB significantly differently from the predominant variant of hemoglobin (Figure 3). This result supported the hypothesis that polymorphisms on the hemoglobin β-chain, which was shown by Kumar et al. to not interact with *S. aureus* hemoglobin receptor IsdH [9], would not significantly alter interaction with IsdB. It is therefore suggested that, within the human population, β-chain polymorphisms likely do not affect infection.

Contrastingly, α-chain polymorphisms decreased the levels of interaction with IsdB. αD6Y, αK7E, αK11Q, αD75Y, and αN78K polymorphisms all significantly decreased the ability of IsdB to bind hemoglobin (all $p<0.001$, Figure 3). These polymorphisms, selected for their proximity to the crucial IsdH interaction sites described by Kumar *et al.* [9], thus support the hypothesis that the interactions of IsdH and IsdB with hemoglobin are structurally homologous. αS3F, αA5D, and αH72Y polymorphisms, likewise selected for their proximity to hemoglobin sites recognized by IsdH, did not induce significant differences in levels of interaction with IsdB (Figure 3). It is probable that αS3, αA5, and αH72 are not critical recognition sites for receptor IsdB; their mutation therefore would not cause differential binding.

The results of the variant screen demonstrated that natural polymorphisms found in human hemoglobin can alter recognition by the *S. aureus* hemoglobin receptor IsdB. It is possible, then, that the hemoglobin of populations possessing the polymorphisms characterized by this research is bound less effectively by IsdB. As a result, these populations may be less susceptible to infection by *S. aureus*. However, it is important to note that most of the polymorphisms found to decrease hemoglobin recognition by IsdB are rare in the human population [8].

Of the identified polymorphisms, only αN78K, which was previously observed in black families from the Congo, Uganda, Zaire, Texas (U.S.), and Alsace (France), is relatively prevalent. It becomes apparent that people with the hemoglobin polymorphisms identified in this study constitute a small minority. Even so, a vast majority of hemoglobin variants still remain uncharacterized with respect to interaction with IsdB. Future research will evaluate the interaction between IsdB and the remaining variants unexamined by this particular study.

The broader generalization of this study is that hemoglobin genotype is a significant factor in staphylococcal infection; its consideration during treatment may prove necessary in the future.

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SUPPORTING INFORMATION.

**Figure S1.** Gel electrophoresis verifying presence of relevant plasmid constructs in vector screen co-transformants

**Figure S2.** Gel electrophoresis verifying presence of relevant plasmid constructs in variant screen co-transformants

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