

Communication

Involvement of H-NS in *Acinetobacter baumannii*'s natural transformation

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Abstract: Most *Acinetobacter baumannii* strains are naturally competent. Although some information is available about factors that enhance or reduce the frequency of transformation of this bacterium, the regulatory elements and mechanisms are barely understood. In this article, we describe studies on the role of H-NS in the regulation of expression of genes related to natural competency and the ability to uptake foreign DNA. The expression levels of the natural transformation-related genes *pilA*, *pilT*, *pilQ*, *comEA*, *comEC*, *comF*, and *drpA* were significantly increased in a Δhns derivative of *Acinetobacter baumannii* A118. Complementation of the mutant with a recombinant plasmid harboring *hns* restored expression levels of six of these genes (*pilT* remained expressed at high levels) to those of the wild-type strain. The transformation frequency of the *A. baumannii* A118 Δhns strain was significantly higher than that of the wild-type. Similar, albeit not identical, effects occurred when *hns* was deleted from the hypervirulent *A. baumannii* AB5075 strain. Reduction of gene expression in a few cases was not as pronounced as to reach wild-type levels, and expression of *comEA* was enhanced further. In conclusion, the expression of all seven transformation-related genes was enhanced after deleting *hns* in *A. baumannii* A118 and AB5075, and these modifications are accompanied by an increase in the cells' transformability. The results demonstrate a role of H-NS in *A. baumannii*'s natural competence.

Keywords: *Acinetobacter baumannii*, H-NS, natural transformation, naturally competent, DNA acquisition

1. Introduction

Histone-like nucleoid structuring protein (H-NS) is a global regulator, widely distributed among different genera of bacteria. H-NS functions to directly repress transcription across the genome. H-NS-like proteins were shown to assist horizontal DNA transmission and have important implications for bacterial evolution [1]. In *Enterobacteriaceae*, H-NS acts as a transcriptional repressor of the type I-E CRISPR-Cas system leading to natural transformation events [2,3]. Eijkelkamp et al. (2013) proposed that H-NS also

demonstrates a xenogeneic repressor role in *A. baumannii*, since a correlation between H-NS-mediated regulation and lack of conservation of the respective potential horizontally acquired gene clusters in different *Acinetobacter* genomes [4]. *A. baumannii* H-NS disruption is known to regulate genes associated with quorum sensing, type VI secretion system, type I pili, phenylacetic acid degradation, acetoin metabolism, among other functions [4]. Horizontal gene transfer (HGT) mechanisms play a crucial role in the dissemination of antimicrobial resistance [5,6]. Natural transformation, which is one of the principle HGT mechanisms is used to integrate exogenous DNA and has been documented in approximately 80 bacterial species [5,7]. Many of the *Acinetobacter* species are naturally competent, which makes transformation a critical strategy for evolution and the acquisition of novel genetic material [8-20]. *A. baumannii*'s genomes are highly variable, showing large segments of DNA of different origin, which often code for virulence factors, adaptability systems, and antibiotic resistance [21-24].

A. baumannii transformation frequency increases in the presence of human pleural fluid and human serum albumin [13,20,25]. Furthermore, *A. baumannii* DNA uptake by *A. baumannii* occurs while moving across wet surfaces [12]. Further studies refined our understanding of natural competency, which shows a correlation with growth phase-dependent synthesis of a type IV pilus [26]. The studies briefly described above conclusively showed that motility and natural competence are intimately associated. Then, it is possible that other factors affecting motility also impact the capability of *A. baumannii* to take up DNA. The recent report that disruption of the *A. baumannii* ATCC 17978 *hns* gene by an insertion sequence results in hyper-motility [27] as well as the role of H-NS in genome stability [1,2] opened the speculation that an H-NS function may also be associated with natural transformation in *A. baumannii*. This manuscript describes significant enhancement in expression levels of genes related to natural competence when *hns* is deleted.

2. Results and Discussion

2.1. H-NS role in natural transformation in the first naturally competent *Acinetobacter baumannii* clinical isolate

To determine the effect of the H-NS global regulator in natural transformation, we deleted the gene in two experimentally validated *A. baumannii* strains and determined the expression of competence-associated genes. Pilus-related genes and twitching motility are essential for *A. baumannii*'s transformability [8,26]. Therefore, we compared the expression levels of *pilA*, *pilT*, *pilQ*, *comEA*, *comEC*, *comF*, and *drpA* in the wild type, the Δhns , and a complemented strains. The latter was constructed by introducing the *hns* carrying plasmid pMBLe-*hns* into the Δhns mutant. Quantitative RT-PCR (qRT-PCR) assays using total RNA showed that expression levels of all tested genes were significantly increased (Fig. 1A). Furthermore, except for *pilT*, the expression levels of these genes in the complemented mutant were reduced to wild-type levels (Fig. 1A). Consistent with these results, assessment of the wild-type and the Δhns mutant transformation frequencies showed a 5-fold increase in *A. baumannii* A118 Δhns ($P < 0.05$) (Fig. 1B).

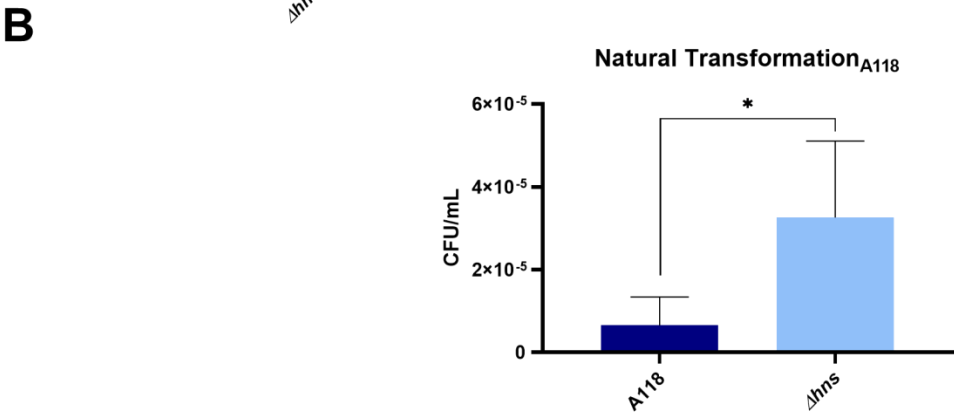
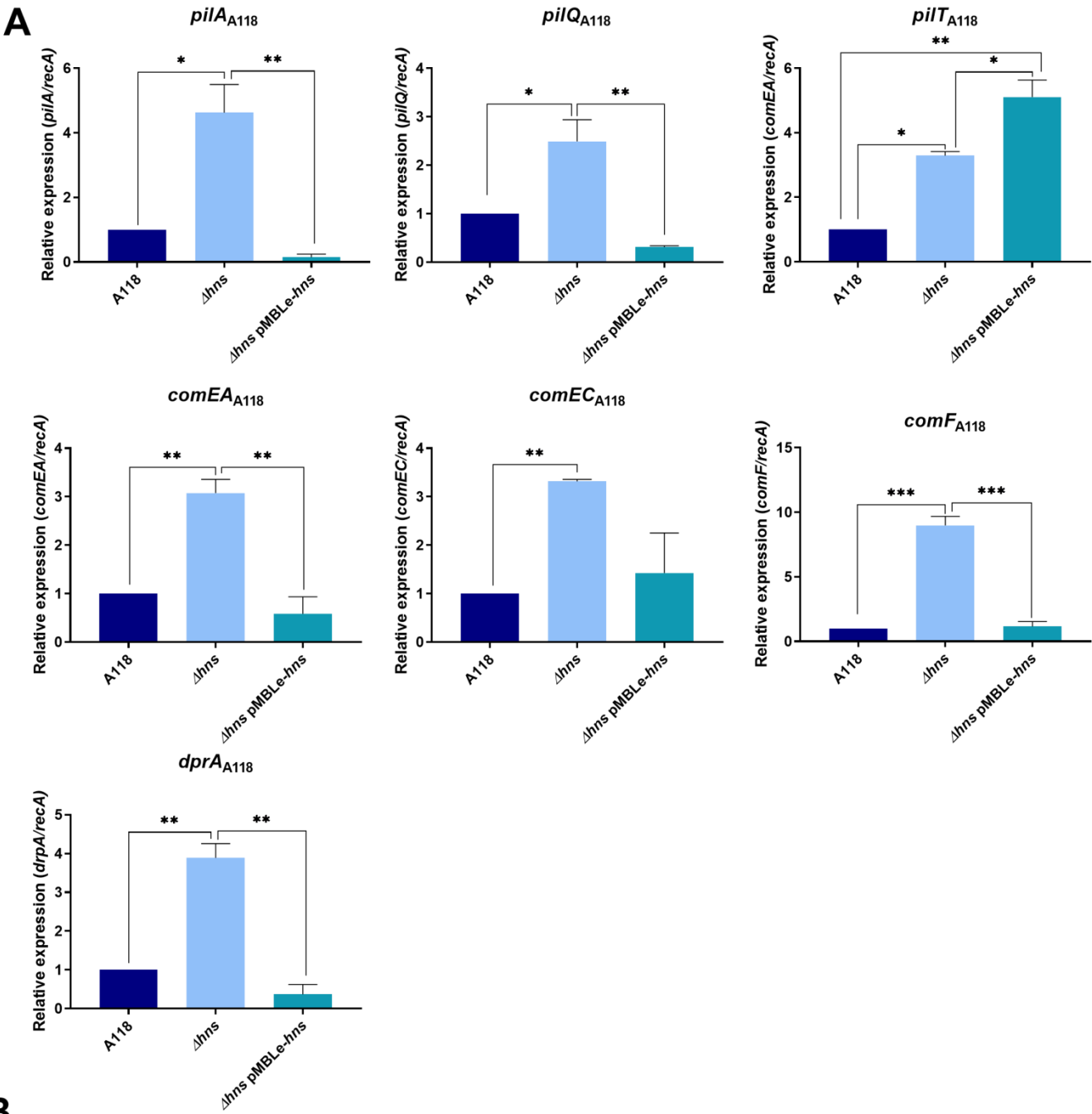


Figure 1. A) qRT-PCR of *A. baumannii* A118, A118 Δhns and A118 Δhns pMBLe-*hns* genes associated with competence and type IV pilus, *pilA*, *pilQ*, *pilT*, *comEA*, *comEC*, *comF* and *drpA*. Fold changes were calculated using double ΔCt analysis. At least three independent samples were tested, and three technical replicates were performed from each sample. Statistical significance ($P < 0.05$) was determined by ANOVA followed by Tukey's comparison test; one asterisk: $P < 0.05$; two asterisks: $P < 0.01$ and three asterisks: $P < 0.001$. B) Natural transformation frequencies for *A. baumannii* A118 and A118 Δhns strains in LB broth. At least three independent replicates were performed and $P < 0.05$ was considered significant (t test).

3.2. The expression of natural competence associated genes is also under the control by H-NS in a hypervirulent and resistant model strains

The role of H-NS in natural competence was also determined in the hypervirulent *A. baumannii* AB5075, its Δhns derivative, and a complemented strain carrying pMBLe-*hns*. As was the case for *A. baumannii* A118 and A118 Δhns , all seven genes, *pilA*, *pilT*, *pilQ*, *comEA*, *comEC*, *comF*, and *drpA*, were expressed at higher levels in the mutant (Fig. 2A). Complementation by the introduction of pMBLe-*hns* was accompanied by a reduction in expression levels in six genes. Only expression of *comEA* deviated from this pattern. As was the case with *pilA* in the *A. baumannii* A118 Δhns (pMBLe-*hns*), we do not know the meaning of these responses to the production of H-NS from an extrachromosomal element. Natural transformation frequency of *A. baumannii* AB5075 Δhns was 5-fold higher than that of the AB5075 parent strain (Fig. 2B).

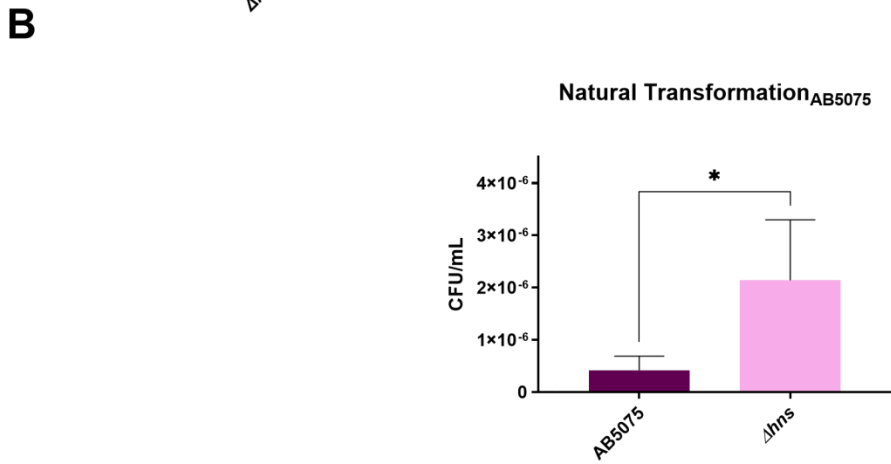
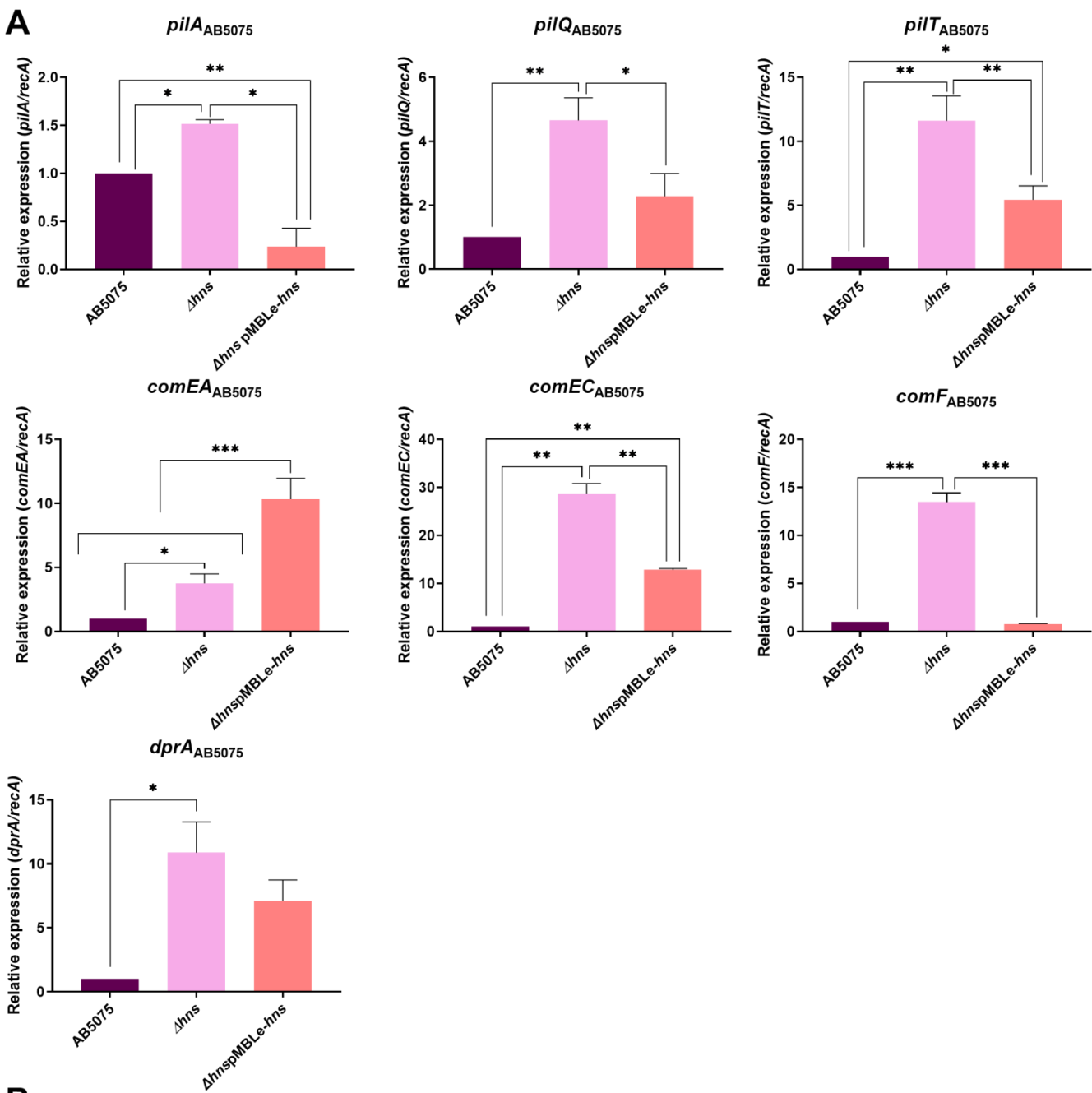


Figure 2 A) qRT-PCR of *A. baumannii* AB5075, AB5075 Δhns and AB5075 Δhns pMBLe-*hns* genes associated with competence and type IV pilus, *pilA*, *pilQ*, *pilT*, *comEA*, *comEC*, *comF* and *dprA*. Fold changes were calculated using double ΔC_t analysis. At least three independent samples were used, and three technical replicates were performed from each sample. Statistical significance ($P < 0.05$) was determined by ANOVA followed by Tukey's comparison test; one asterisks: $P < 0.05$; two asterisks: $P < 0.01$ and three asterisks: $P < 0.001$. B) Natural transformation frequencies for *A. baumannii* AB5075 and Δhns strains in LB broth. At least two independent replicates were performed and $P < 0.05$ was considered significant *t* test.

3. Materials and Methods

3.1. Bacterial strains

The model susceptible *A. baumannii* A118, the isogenic A118 Δhns mutant, and A118 Δhns containing the plasmid pMBLe-*hns*, which expresses a wild-type copy of *hns* under the control of own promoter were used (Rodgers et al. 2021, submitted). In addition, to extend H-NS role on *A. baumannii* response, the multidrug and hypervirulent *A. baumannii* AB5075 strain, AB5075 Δhns [28], and AB5075 Δhns pMBLe-*hns* were used in the present study.

3.2. RNA extraction and qRT-PCR

A. baumannii A118 and AB5075 and their derivative strains were cultured in lysogeny broth (LB) and incubated with agitation for 18 h at 37 °C. Overnight cultures were then diluted 1:10 in fresh LB broth and incubated with agitation for 7 h at 37 °C. The Direct-zol RNA Kit (Zymo Research, Irvine, CA, USA) was used to perform the RNA extraction (in triplicate). RNA samples were treated with DNase (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instruction. Samples were confirmed to have no DNA contamination through PCR amplification of the 16S rDNA gene. qRT-PCR was next performed to analyze the expression of natural transformation associated genes. cDNA was prepared using the iScript™ Reverse Transcription Supermix for qRT-PCR (BioRad, Hercules, CA, USA) and quantitative PCR was performed using iQ™SYBR® Green Supermix (BioRad, Hercules, CA, USA) per the manufacturer's recommendations, respectively. Results were analyzed using the $2^{-\Delta\Delta C_t}$ method in which *recA* [29] acted as the control gene. Experiments were performed in technical and biological triplicate and statistical analysis (ANOVA followed by Tukey's comparison test) was performed using GraphPad Prism (GraphPad software, San Diego, CA, USA). A *P-value* < 0.05 was considered significant.

3.3. Natural transformation assays

Natural transformation assays were performed as were previously described [25,26]. Briefly, 20 μ l of *A. baumannii* cells grown overnight in LB medium at 37°C were mixed with 1 μ g of pMBLe-OA-ArK (apramycin resistance) plasmid and the mixture was spotted onto twitching motility plates (10). After 4 h of incubation at 37 °C, the cells were scraped off from the plate and resuspended in a microcentrifuge tube containing 200 μ L of LB medium. Transformation events were scored by counting apramycin (15 μ g/mL) colonies, while total colony forming units (CFUs) was assessed by plating serial dilutions on LB agar plates. Negative controls with no DNA addition were included in every tested condition. All experiments were performed in triplicate and statistical analysis was performed. Transformation events were scored as mentioned above. Experiments were performed in technical and biological triplicate and statistical *t* test analysis was performed using GraphPad Prism (GraphPad software, San Diego, CA, USA). A *P-value* < 0.05 was considered significant.

4. Conclusions

The global repressor H-NS modulates the expression of a plethora of *A. baumannii* genes with functions related to virulence, biosynthetic pathways, cell adhesion, quorum sens-

ing, and autotransporters, among others. The focused analysis of the role of H-NS in the regulation of expression of genes related to mobility and DNA transformation carried out in this work showed that a) seven genes that code functions associated with natural competence are overexpressed in the absence of H-NS, and b) transformation frequency is higher in the absence of this protein. Consequently, H-NS modulates the DNA uptake by *A. baumannii* strains, suggesting a participation in gene acquisition and concomitantly evolution during infectious processes.

Author Contributions: C.L., C.P., M.R.T., T.S., J.E., B.N., S.A., D.R., R.A.B., R.S., M.E.T. and M.S.R. conceived the study and designed the experiments. C.L., C.P., M.R.T., T.S., J.E., B.N., S.A., D.R., R.S., and M.S.R. performed the experiments and genomics and bioinformatics analyses. M.R.T., T.S., R.S., R.A.B., M.E.T. and M.S.R. analyzed the data and interpreted the results. R.A.B., M.E.T. and M.S.R. contributed reagents/materials/analysis tools. M.R.T., T.S., R.S., R.A.B., M.E.T. and M.S.R. wrote and revised the manuscript. All authors read and approved the final manuscript.

Funding: The authors' work was supported by NIH SC3GM125556 to MSR, R01AI100560 to RAB, R01AI063517, R01AI072219 to RAB and 2R15 AI047115 to MET. CP and JE were supported by grant MHRT 2T37MD001368 from the National Institute on Minority Health and Health Disparities, National Institute of Health. DR has a MARC U*STAR fellowship by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number T34GM008612. SA was supported by Project RAISE, U.S. Department of Education HSI-STEM award number P031C160152. This study was supported in part by funds and/or facilities provided by the Cleveland Department of Veterans Affairs, Award Number 1I01BX001974 to RAB from the Biomedical Laboratory Research & Development Service of the VA Office of Research and Development and the Geriatric Research Education and Clinical Center VISN 10 to RAB. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the Department of Veterans Affairs. MRT and TS are recipient of a post-doctoral fellowship from CONICET. R.S. is a staff member from CONICET.

Conflicts of Interest: The authors declare no conflict of interest.

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