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Regulation of *Helicobacter pylori* adherence by gene conversion

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Summary

Genetic diversification of *Helicobacter pylori* adhesin genes may allow adaptation of adherence properties to facilitate persistence despite host defenses. The *sabA* gene encodes an adhesin that binds sialyl-Lewis antigens on inflamed gastric tissue. We found variability in the copy number and locus of the *sabA* gene and the closely related *sabB* and *omp27* genes due to gene conversion among 51 North American pediatric *H. pylori* strains. We determined that *sabB* to *sabA* gene conversion is predominantly the result of intra-genomic recombination and RecA, RecG, and AddA influence the rate at which it occurs. Although all clinical strains had at least one *sabA* gene copy, *sabA* and *sabB* were lost due to gene conversion at similar rates in vitro, suggesting host selection to maintain the *sabA* gene. *sabA* gene duplication resulted in increased SabA protein production and increased adherence to sialyl-Lewis antigens and mouse gastric tissue. In conclusion, gene conversion is a mechanism for *H. pylori* to regulate *sabA* expression level and adherence.

Keywords

Helicobacter pylori; *sabA* gene; adhesin; adherence; gene conversion; genetic diversity

Introduction

Helicobacter pylori, which infects the stomach of over half the world's population, is generally acquired in childhood and can persist for the life of the infected host by evading host immune defenses and adapting to changing conditions in the stomach. *H. pylori* causes persistent gastritis (inflammation of the gastric mucosa) in all who are infected and this eventually leads to peptic ulcers and gastric cancer in a subset of infected individuals.

H. pylori exhibits a high level of genetic diversity, and genetic differences among *H. pylori* strains contribute to the differences in disease outcome among infected patients (Atherton *et al.*, 1995; Blaser *et al.*, 1995; Covacci *et al.*, 1993; Gerhard *et al.*, 1999; Nomura *et al.*, 2002). Furthermore, genetic changes during the course of infection could allow *H. pylori* to adapt and persist in the face of host defenses, and thus would have an important role in the ability of *H. pylori* to maintain a life-long infection.

Genetic changes in *H. pylori* genes that encode outer membrane proteins (*omp* genes) are particularly likely to facilitate adaptation of *H. pylori* to the changing environment in the host. There are over 60 *omp* genes in the *H. pylori* genome and some are known to be adhesins (Ilver *et al.*, 1998; Mahdavi *et al.*, 2002; Odenbreit *et al.*, 1999). The middle

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regions of the *omp* genes are variable, but the 5′ and 3′ ends are conserved. In some cases, the percent nucleotide identity at the 5′ and 3′ ends is high enough to allow for recombination between members of the gene family that result in gene conversions and switching of loci. This has been observed among the *omp* gene *babA*, which encodes the adhesin that binds Lewis[b] antigens on gastric tissue, and two closely related *omp* genes, *babB* and *babC* (Colbeck *et al.*, 2006; Hennig *et al.*, 2006; Pride and Blaser, 2002).

The *omp* gene *sabA* (*omp17*, *hopP*) encodes the adhesin that binds sialyl-Lewis antigens that are expressed on inflamed gastric tissue (Mahdavi *et al.*, 2002). *H. pylori* infection induces gastritis and then is able to exploit receptors expressed by the inflamed tissue for close adherence. Adherence to the gastric epithelium can be beneficial to *H. pylori* because it helps the bacteria to avoid clearance and brings them closer to nutrients. However, adherence can also be detrimental in the presence of host immune responses that are most intense at the gastric epithelial surface. Therefore, *H. pylori* adherence properties may need to be dynamic for *H. pylori* to maintain a stable colonization. Genetic diversification of adhesin genes could allow *H. pylori* to adapt by constantly generating new variants.

The 5′ and 3′ ends of *sabA* share the highest nucleotide identity (80–100%) with two other *omp* genes, *sabB* (*omp16*, *hopO*) and *omp27* (*hopQ*). Although the function of the *sabB* and *omp27* genes is not known, both may also have a role in adherence (Loh *et al.*, 2008; Yamaoka *et al.*, 2002). Comparison of the gene content of fully sequenced *H. pylori* strains from different global regions found *sabA*, *sabB*, and *omp27* copy number variability (Kawai *et al.*, 2011), which appears could have resulted from recombination among these three genes. SabA and SabB expression can be switched on and off by slipped-strand mispairing in a 5′ dinucleotide repeat region, allowing translational phase variation (Goodwin *et al.*, 2008). Genetic diversification resulting from recombination among the *sabA*, *sabB*, and *omp27* genes could be another mechanism for *H. pylori* to regulate its adherence properties.

To better understand how *H. pylori* employs genetic variation to regulate production of the SabA adhesin, we investigated the copy number and locus of the *sabA* gene among North American pediatric *H. pylori* strains and found evidence of recombination with the *sabB* and *omp27* genes. To explore the consequences of *sabA* gene duplication, we examined SabA protein production and binding to sialyl-Lewis[x] (sLex) as well as mouse gastric tissue in isogenic strains with gene configurations observed in our clinical strain collection. We also measured and compared the rates of gene conversion at the *sabA*, *sabB*, and *omp27* loci in vitro and investigated the role of natural competence and recombination proteins as potential mediators of these gene conversion events.

Results

Variability in copy number and locus of sabA, sabB, and omp27 genes among clinical H. pylori strains

The gene content of the *sabB* (*jhp0659*), *sabA* (*jhp0662*), and *omp27* (*jhp1103*) loci (as defined by the *H. pylori* reference strain J99) was characterized for 51 of the 53 clinical *H. pylori* strains collected in a study of North American pediatric strains (Talarico *et al.*, 2009) (Figure 1). Two strains (both isolated from the same patient) failed to produce PCR product despite sufficient DNA quantity and quality. Of the 51 characterized strains, 10 (20%) had the *sabB* gene at the *sabB* locus, 23 (45%) did not have a gene at the *sabB* locus, and 18 (35%) had either the *sabA* or *omp27* gene at the *sabB* locus. At the *sabA* locus, 43 (84%) of the 51 strains had *sabA* and 8 (16%) had either *sabB* or *omp27*. All 51 strains had the *omp27* gene at the *omp27* locus. The *sabB* gene was absent from the genome of 35 (69%) of the 51 strains.

Sequence comparison suggests recent intra-strain gene conversion events

For strains having two identified copies of the *sabA* or *omp27* gene, the two copies were sequenced and compared to examine whether the second copy resulted from an intra-strain gene conversion event, indicated by a high level of nucleotide identity. Of the four strains with two identified *sabA* genes, two strains appeared to have had a recent intra-strain gene conversion event. One strain had two *sabA* genes that differed by one dinucleotide repeat and the other strain had two *sabA* genes that differed by 11 SNPs, all within the last 140 bp. The other two strains had two divergent *sabA* genes (126 and 304 SNPs, 4 and 26 indels, respectively), suggesting that either one copy resulted from recombination with a distantly related allele, perhaps from a super-infecting strain via natural transformation, or that gene conversion occurred relatively longer ago followed by sequence divergence. Of the eight strains that had two identified *omp27* genes, six had one Type I allele and one Type II allele, two highly divergent allele types (Cao and Cover, 2002), indicating that one *omp27* gene was not a duplication of the other. The other two strains both had two copies of an *omp27* Type I allele that appeared to be the result of a recent intra-strain gene conversion event. One strain had two identical *omp27* genes and the other strain had two *omp27* genes that differed by four SNPs.

Gene conversion occurs at a similar rate at the sabA and sabB loci and at a lower rate at the omp27 locus in vitro

In the clinical strains, gene conversion involved duplication of the *sabA* or *omp27* gene and loss of the *sabB* gene. This could be due to homologous recombination being favored at the *sabB* locus or to host selective pressures to preserve the *sabA* and *omp27* genes. To investigate whether the pattern of gene conversion in the clinical strains is due to homologous recombination being favored at the *sabB* locus, the rate of gene conversion at the *sabA*, *sabB*, and *omp27* loci in the absence of selection were compared by fluctuation analysis. For these studies the unique regions of *sabA, sabB* or *omp27* were replaced by a cassette allowing both positive (*cat*; chloramphenicol resistance) and negative (*sacB*, sucrose sensitivity) selection. We grew parallel cultures in non-selective media starting from a very small number of cells for ~24 generations and then identified clones having spontaneous gene conversion (Figure 2A). *sabA* duplication at the *sabB* locus and *sabB* duplication at the *sabA* locus occurred at a similar rate, indicating that the *sabA* gene can be readily lost due to gene conversion in the absence of selective pressures. We did not observe *omp27* duplication in any experiment and gene conversion at the *omp27* locus was not observed in any of 29 cultures examined (Table 3, Figure 2B). Our in vitro gene conversion experiments were performed in strain NSH57, a derivative of the sequenced human clinical isolate G27 (Baldwin *et al.*, 2007; Baltrus *et al.*, 2009). To explore the generality of our findings, we repeated the gene conversion experiment in a second clinical isolate, J99 (Alm *et al.*, 1999). The rate of *sabA* gene conversion at the *sabB* locus was similar between the J99 and NSH57 strains (Table 3, Figure 2B).

sabA gene conversion predominantly results from intra-genomic recombination and occurs at a rate influenced by RecA, AddA, and RecG

Since *H. pylori* is naturally competent for the uptake of exogenous DNA, *sabA* gene conversion may result from natural transformation with DNA from neighboring *H. pylori* cells. To assess the role of natural competence in *sabA* gene conversions, the rate of *sabA* gene conversion at the *sabB* locus of a *comB10* mutant, which cannot take up exogenous DNA for natural transformation, was compared to that of wild-type. The *comB10* mutant had a *sabA* gene conversion rate similar to that of wild-type (Table 3, Figure 2B) suggesting that *sabA* gene conversion is largely the result of intra-genomic recombination in the in vitro assay. To investigate the mediators of homologous recombination in *sabA* gene conversions, the rate of *sabB* to *sabA* gene conversion in a *recA* mutant, an *addA* mutant, and a *recG*

mutant was compared to that of wild-type (Table 3, Figure 2B). RecA, which facilitates homology-dependent strand exchange, and AddA, a nuclease-helicase involved in double-strand break repair, have been shown to promote *babA* to *babB* gene conversion (Amundsen *et al.*, 2008) and RecG has previously been implicated in limiting both inter-genomic and intra-genomic recombination (Kang *et al.*, 2004). The *recA* mutant had a *sabA* gene conversion rate ~4-fold lower than that of wild-type, although *sabA* gene conversion was still observed indicating these recombination events can also be RecA-independent. The *addA* mutant had a *sabA* gene conversion rate ~14-fold lower and significantly different than that of wild-type, suggesting that these gene conversion events may be initiated by a double-strand break. The *recG* mutant had a *sabA* gene conversion rate ~40-fold higher and significantly different than that of wild-type, further supporting the role of RecG in limiting recombination.

Duplication of the sabA gene results in increased production of the SabA adhesion

sabA gene duplication that results in the second copy being expressed from the *sabB* promoter could result in SabA protein production that is less than or greater than two-fold because of nucleotide sequence differences between the *sabB* and *sabA* promoters including differences in the length of a poly-thymine tract. We thus investigated whether *sabA* expression levels varied depending on whether the *sabB* promoter was preserved during the recombination event that generated the second copy of *sabA* at the *sabB* locus. *sabA* expression level was measured by qRT-PCR for an NSH57 variant clone (ST4) and two J99 variant clones (ST83 and ST100) that had two *sabA* genes with the second copy at the *sabB* locus with the *sabB* promoter, and a J99 variant clone (ST101) that had two *sabA* genes with the second copy at the *sabB* locus with the *sabA* promoter. The NSH57 and J99 variant clones were selected from a small subset of chloramphenicol-resistant and sucrose-sensitive clones that arose during the gene conversion experiments for which frozen stocks were made prior to confirming a gene conversion event. Two *sabA* copies resulted in approximately two-fold expression relative to the parental strain regardless of whether the second copy was expressed from the *sabA* or *sabB* promoter in both the NSH57 and J99 strain backgrounds (ST4:2.3(2.1–2.6), ST83:1.6(1.1–2.5), ST100:1.6(1.0–2.8), ST101:2.0(1.2–3.1); fold-change(range)).

Given that gene conversions resulting in a second copy of the *sabA* gene resulted in two-fold higher *sabA* message, regardless of the promoter sequence driving *sabA* at the *sabB* locus, we wondered whether *sabA* gene duplication could be a mechanism for *H. pylori* to vary the amount of SabA adhesin produced. We were only able to test this in the NSH57 strain background because in the J99 variants one or both *sabA* genes in these strains were out of frame due to dinucleotide repeat changes within the 5′ end of the coding sequence that altered the reading frame. Immunoblotting was used to compare SabA protein production between NSH57 and an NSH57 variant clone (ST22) that arose during the gene conversion rate experiments. ST22 has two in-frame copies of the *sabA* gene both controlled by the *sabA* promoter, with the second *sabA* gene copy at the *sabB* locus. As expected, ST22 produced twice as much SabA protein as wild-type NSH57 (adjusted relative SabA density: 2.07±0.15, mean of three immunoblots ± standard deviation, Figure 3).

Duplication of the sabA gene results in increased adherence to gastric tissue

Varying the amount of SabA adhesin produced may allow *H. pylori* to adapt its adherence properties depending on the selective pressures in the host environment. To test whether a two-fold increase in SabA adhesin has an effect on *H. pylori* adherence to gastric tissue, an in vitro binding assay of *H. pylori* to mouse gastric tissue sections was performed. Previous evidence has suggested that *H. pylori* adherence to mouse gastric tissue is mediated by both the SabA adhesin and the BabA adhesin, which binds to host Lewis[b] antigens, although

mice are known to lack an alpha-1,3/4 fucosyltransferase needed for biosynthesis of Lewis[b] (Magalhaes *et al.*, 2009). Because BabA-mediated binding could potentially mask the adherence effects of a two-fold increase in SabA adhesin, we disrupted the *babA* gene of NSH57 and ST22 as well as a *sabA* deletion mutant to make ST164 (one *sabA* copy, *babA* mutant), ST166 (two *sabA* copies, *babA* mutant), and ST168 (*sabA* and *babA* mutant), respectively. ST168 showed an 86% decrease in adherence compared to ST164 demonstrating that the SabA adhesin contributes to adherence to mouse gastric tissue (ST164: 0.0148 ± 0.0026 adherent bacteria per μm^2, mean of 8 regions of tissue $\pm$ standard deviation; ST168: 0.0020 ± 0.0006 adherent bacteria per μm^2, mean of 5 regions of tissue $\pm$ standard deviation; Student's t-test, p< 0.0001). This is further supported by an 89% decrease in adherence when ST164 is preincubated with sLex (ST164 with 1 ng μl-1 free sLex: 0.0017 ± 0.0004 adherent bacteria per μm^2, mean of 5 regions of tissue $\pm$ standard deviation; Student's t-test to compare to ST164, p<0.0001; Figure 4A–B). By incubating the tissue with different concentrations of *H. pylori* (OD_{600} 0.1, 0.05, 0.01, and 0.001), we saw that adherence of ST164 and ST166 becomes saturated at OD_{600} 0.1 and that a difference in the number of adherent bacteria can be seen at OD_{600} 0.001 and OD_{600} 0.01 (Figure 4C). When the tissue was incubated with *H. pylori* at OD_{600} 0.001, the strain having two copies of *sabA* (ST166) had a 250% increase in adherent bacteria compared to the strain ST164 having one *sabA* copy (ST164: 0.0002 ± 0.0001 adherent bacteria per μm^2, ST166: 0.0007 ± 0.0005 adherent bacteria per μm^2; mean of two experiments $\pm$ standard deviation, Student's t-test, p = 0.002). When the tissue was incubated with *H. pylori* at OD_{600} 0.01, ST166 had a 39% increase in binding compared to ST164 (ST164: 0.0028 ± 0.0009 adherent bacteria per μm^2, ST166: 0.0039 ± 0.0024 adherent bacteria per μm^2; mean of two experiments $\pm$ standard deviation, Student's t-test, p = 0.125). Because the magnitude of the increase in adherence at OD_{600} 0.01 is small and the variation is large, we evaluated the difference in adherence at this bacterial concentration in further experiments to determine whether the difference was statistically significant. When we increased the sample size, the strain having two *sabA* copies (ST166) showed a 44% increase in adherence compared to the strain having one *sabA* copy (ST164) and this difference reached statistical significance (ST164: 0.0025 ± 0.0011 adherent bacteria per μm^2, ST166: 0.0036 ± 0.0020 adherent bacteria per μm^2; mean of 5 experiments $\pm$ standard deviation, Student's t-test, p = 0.008; Figure 4D). These experiments show that, at both the lower concentrations of bacterial cells added, *H. pylori* having two-fold greater SabA protein production resulting from *sabA* gene conversion show an increase in adherence to mouse gastric tissue.

Duplication of the sabA gene results in increased adherence to sialyl-Lewis[x]

In the stomach Lewis antigens and sialyl-Lewis antigens are expressed on the epithelial cell surface proteins and lipids as well as secreted mucin molecules. In fact cleavage and release of cell associated mucin molecules may be an innate immune strategy to remove bacteria from the epithelium (Linden *et al.*, 2009). To explore the effects of a two-fold increase in SabA adhesin on binding to surface associated sialyl-Lewis antigens in the presence of soluble sialyl-Lewis antigens, an ELISA-based adherence assay with immobilized sLex-coated wells was performed. In the absence of soluble sLex, NSH57 and ST22 both adhered to the immobilized sLex but no significant difference in adherence was detected (adherence ratios: NSH57 1.87 ± 0.4, ST22 1.91 ± 0.4; mean of 5 experiments $\pm$ standard deviation; Student's t-test, p=0.87). Inhibition of *H. pylori* binding by addition of free sLex was then compared between NSH57 and ST22. Inhibition of NSH57 adherence increased with increasing concentrations of free sLex added (Figure 5A). Addition of 10 ng μl^{-1} free sLex resulted in a $41\% \pm 10\%$ decrease (mean of four experiments $\pm$ standard deviation) in NSH57 adherence but no decrease in ST22 adherence (Figure 5B) indicating that a two-fold increase in SabA adhesin resulting from *sabA* gene duplication increases *H. pylori* adherence to immobilized sialyl-Lewis[x].

Discussion

We measured variability in the copy number and locus of the *sabA* gene among pediatric clinical *H. pylori* strains and among clones of a single strain resulting from recombination with the closely related *sabB* and *omp27* genes. Changes in *sabA* copy number generate subpopulations of bacterial cells having differences in SabA adhesin production level that alters adherence to surface associated sialyl-Lewis antigens in the presence of soluble sialyl-Lewis antigens and to gastric tissue. While it has been shown that *sabA* expression can be turned on or off by translational phase variation (Mahdavi *et al.*, 2002), *sabA* gene duplication by gene conversion represents an additional mechanism for regulating the amount of SabA protein produced on the bacterial surface. The ability of *H. pylori* to modulate its adherence properties is important since there are benefits and risks associated with adherence to the host epithelium. *H. pylori* may persistently infect the stomach by maintaining a balance between an adherent population and a population in the mucus layer.

We found that *H. pylori* having two-fold greater SabA protein production resulting from *sabA* gene conversion show an increase in adherence to mouse gastric tissue. To improve our ability to detect a difference in adherence, these experiments were performed in a *babA* mutant background because a previous study has shown that *H. pylori* adherence to mouse gastric tissue is mediated by both the SabA adhesin and the BabA adhesin despite the fact that mice are believed to lack the transferases necessary to synthesize Lewis[b] (Magalhaes *et al.*, 2009). SabA protein production may be especially important in the absence of BabA-mediated adherence. BabA-mediated binding is absent in many human infections either due to the *H. pylori* strain lacking the *babA* gene or carrying a nonfunctional *babA* allele (Gerhard *et al.*, 1999; Hennig *et al.*, 2004) or the host not expressing the Lewis[b] antigen (Sakamoto *et al.*, 1989). A study of *H. pylori* clinical isolates that uniformly expressed BabA found that SabA expression was associated with greater *H. pylori* density but only in patients having weak or no Lewis[b] expression (Sheu *et al.*, 2006). Furthermore, expression of fucosylated antigens such as Lewis[b] were shown to reciprocally decrease while sialylated antigens increase during persistent *H. pylori* infection of Rhesus monkeys due to competition for carbohydrate chains by the sialyl and fucosyl transferases (Linden *et al.*, 2008). Thus, SabA and BabA-mediated adherence may be important at different times during persistent *H. pylori* infection. These observations also suggest that variation of *H. pylori* adherence properties may allow this organism to adapt to changing selective pressures in the gastric environment including shifts in the available host receptors over time or upon transmission to a new host.

We also demonstrated that two-fold greater SabA protein production resulted in an increase in adherence to immobilized sialyl-Lewis[x] but only in the presence of free sialyl-Lewis[x]. This is biologically relevant because sialyl-Lewis antigens, in addition to being expressed by the gastric epithelium, are also present on mucin glycoproteins in the mucus layer of the stomach. Mucins are synthesized by the mucosal epithelial cells and can limit *H. pylori* colonization by steric hindrance as well as acting as a releasable decoy when shed into the mucus layer (Linden *et al.*, 2009; McGuckin *et al.*, 2007). Mucins containing sialyl-Lewis antigens in the mucus layer could limit *H. pylori* adherence to the host epithelium by competing with sialyl-Lewis antigens on the epithelial cells for binding to the SabA adhesin. Increasing SabA adhesin production by gene conversion may be a way for *H. pylori* to overcome this competitive binding and better adhere to host tissue.

We estimated the rate of *sabA* gene conversion at the *sabB* locus to be 1.4×10^{-9} gene conversions per cell per generation, similar to the mutation rates that have been reported for rifampicin resistance (2.98×10^{-8} per cell per division) and streptomycin resistance (4.34×10^{-10} per cell per division) in *H. pylori* (Baltrus *et al.*, 2008). A previous study measured the

frequency of *babB* gene conversion at the *babA* locus and estimated from that the rate of *babA* to *babB* gene conversions to be 3×10^{-6} gene conversions per cell division (Amundsen *et al.*, 2008). It is not clear whether the difference in *sabA* and *babB* gene conversion rates is due to differences in experimental methods or whether recombination between *babA* and *babB* occurs at a much higher rate than recombination between *sabA* and *sabB*.

While we observed that *sabA* and *sabB* are lost due to gene conversion at a similar rate in the absence of selection pressures in vitro, the *sabB* gene was lost due to an apparent gene conversion in 12 (24%) of the 51 clinical strains whereas all clinical strains had at least one *sabA* gene copy. This discrepancy suggests there are selective pressures during human infection to maintain the *sabA* gene. However, other studies have found that the *sabA* gene is not present in all strains. Twenty percent of 145 Taiwanese strains and 9% of 23 Japanese strains were *sabA* geno-negative (Shao *et al.*, 2010; Sheu *et al.*, 2006). This could be due to geographic differences among strains or different requirements for retention of *sabA* between adult and pediatric strains.

Interestingly, 45% of the clinical *H. pylori* strains did not have an *omp* gene at the *sabB* locus and thus would be less able to regulate *sabA* expression by gene conversion. This suggests that the genetic mechanisms for regulating the SabA adhesin, which binds sialyl-Lewis antigens produced during inflammation (Linden *et al.*, 2008; Mahdavi *et al.*, 2002), may be adapted to the pro-inflammatory nature of the strain. For example, having no *omp* gene at the *sabB* locus was correlated with absence of the inflammation-promoting *cag* pathogenicity island (PAI) (chi-square test, p<0.0001) in this North American pediatric population. In contrast, recent work showed 9/11 Asian strains lack *sabB* in spite of uniform presence of the *cag* PAI (Kawai *et al.*, 2011). Although it might be possible for the *sabA* gene to duplicate by recombining at some other *omp* locus in the genome, this was not observed in these fully sequenced strains. Asian strains show near universal carriage of a *cag* PAI with enhanced cellular activity compared to *cag* PAI of Western strains (Hatakeyama and Higashi, 2005). Future studies are thus needed to examine the competitive advantage of strains having different available genetic mechanisms for regulating *sabA* expression, especially in the context of *H. pylori* strain differences in induction of inflammation.

Since the *H. pylori* genome encodes relatively few proteins that regulate transcription, genetic diversification is likely an important mechanism for *H. pylori* to modulate phenotypic properties in response to changing conditions in the stomach. This study improves our understanding of how *H. pylori* employs genetic diversification to adapt adherence properties and cause persistent active infection for decades despite a robust host response.

Experimental Procedures

Strain collection

The study sample consisted of 53 *H. pylori* strains isolated from 47 North American pediatric patients and was previously described (Talarico *et al.*, 2009). Briefly, gastric biopsies from the antrum of the stomach were obtained during a diagnostic fiberoptic upper endoscopy performed at the discretion of the pediatric gastroenterologist because of the subjects' persistent gastrointestinal symptoms. Culturing of biopsy samples was performed and single colonies were isolated and typed by RAPD-PCR fingerprinting. Informed consent was obtained from patients or their parents or guardians.

PCR and DNA Sequencing

The gene content of the *sabB* (jhp0659), *sabA* (jhp0662), and *omp27* (jhp1103) loci of the 53 clinical *H. pylori* strains was examined by PCR using a combination of primer pairs in which one primer is specific for the gene flanking the locus and the other primer is inside and specific to the *sabB*, *sabA*, or *omp27* gene (Table 1) and/or by PCR of the entire locus followed by DNA sequencing. The *dcuA* (jhp0660) and *ansB* (jhp0661) genes are inverted in some strains in comparison to the J99 reference genome. In these strains, the jhp0658F and the jhp0661F primers were used to amplify the *sabB* locus and the jhp0660R and jhp0663R primers were used to amplify the *sabA* locus. PCR with primers inside the *sabB* gene (sabBFor and sabBRev) was used to confirm absence of the *sabB* gene in the genome.

Prior to DNA sequencing, PCR products were purified using the Zymo Research DNA Clean and Concentrator™-5 Kit (Zymo Research). Sequencing was performed by the FHCRC Genomics Shared Resource and results were analyzed using FinchTV Version 1.4.0 (Geospiza) and BLAST (NCBI).

Construction of engineered strains and growth conditions

H. pylori knock-out mutants were generated as previously described (Humbert and Salama, 2008) using a vector-free allelic replacement strategy. Briefly, to generate knock-out mutants, 67–98% of the coding sequence of a gene was replaced by either a *cat-sacB* cassette, conferring chloramphenicol resistance and sucrose sensitivity, or the *aphA3* cassette, conferring kanamycin resistance, by natural transformation with PCR product. The genotype of all mutants was confirmed by PCR and/or DNA sequencing. *H. pylori* strains (Table 1) were grown as previously described (Humbert and Salama, 2008).

Fluctuation analysis

To measure the gene conversion rate at the *sabB*, *sabA*, and *omp27* loci, fluctuation analysis (Foster, 2006) was performed using *H. pylori* strains NSH57 (a derivative of G27) and J99 (Alm *et al.*, 1999; Baldwin *et al.*, 2007; Baltrus *et al.*, 2009) engineered with a *cat-sacB* cassette in place of the unique middle region of either the *sabB*, *sabA*, or *omp27* gene (Figure 2A). Between 277 and 368 bp of the 5′ and 3′ homologous ends were left to allow recombination to occur. Twenty cultures of the engineered strain were grown from ~10 cells in 350 µl Brucella broth containing 10% fetal bovine serum in a 24 well plate for 72 hours. A 10 µl sample was taken from four cultures and titred onto non-selective plates to determine the final CFU. The 20 cultures were each plated onto media containing 6% sucrose and sucrose-resistant colonies were then replica plated on non-selective media and media containing 15 µg ml^{-1} chloramphenicol. To confirm replacement of the *cat-sacB* cassette with a duplicated copy of an *omp* gene, chloramphenicol-sensitive and sucrose-resistant clones were first subject to PCR with primers in the flanking genes. For those having a PCR product size consistent with an *omp* gene in the locus, gene conversion was confirmed either by DNA sequencing or by PCR with one primer specific for the gene flanking the locus and the other primer specific for either *sabA*, *sabB*, or *omp27*, as appropriate. Gene conversion rates were calculated using the p_0 method (Foster, 2006). Because a statistical test to compare the gene conversion rates is not available, differences in the gene conversion rates were assessed by comparing the 95% confidence intervals for overlap (Gould *et al.*, 2007; Shaver and Sniegowski, 2003). For each strain, fluctuation analysis was performed at least twice. If the 95% confidence intervals were overlapping, the two rate estimates of the biological replicates were considered not significantly different and the data from the two experiments was combined. The rates of gene conversion at the different loci and in the different strain backgrounds were compared using the 95% confidence intervals for the gene conversion rate obtained from the combined data.

Immunoblotting

To detect SabA protein production, immunoblotting was performed as previously described (Pinto-Santini and Salama, 2009) except the primary antibody was SabA-specific antiserum (Sheu *et al.*, 2006) used at a 1:10,000 dilution and the secondary antibody was goat anti-rabbit conjugated to horseradish peroxidase (Pierce) used at a 1:50,000 dilution. To adjust the relative amounts of SabA protein produced to the amount of protein loaded, the blots were stripped and re-probed with anti-Cag3 as the primary antibody. The relative densities of the SabA and Cag3 bands were determined using the ImageJ software (http://rsb.info.nih.gov/ij/) and SabA band density was normalized to Cag3 band density to compare samples. The mean adjusted relative SabA density was calculated from three technical replicates.

Reverse transcription quantitative PCR (RT-qPCR)

For RNA isolation, liquid cultures were grown to optical density at 600 nm (OD600) 0.4, then collected on 0.45 μm pore size membrane filters (Millipore) and frozen at $-80°C$ prior to RNA extraction, which was performed as previously described (Merrell *et al.*, 2003). Approximately 1 μg RNA was reverse transcribed in a standard reaction with Superscript II (Invitrogen) and 100 μM random octamer primers (Fisher). The resulting cDNA was then used for qPCR, which was performed in a standard reaction using SYBR green on an ABI prism 7900HT sequence detection system (Applied Biosystems). The primers used for qPCR are listed in Table 2. The quantity of *sabA* cDNA was normalized to the quantity of JHP0298 cDNA to compare samples. The fold-difference in *sabA* cDNA of the isogenic *sabA* gene variants were compared using the $\Delta\Delta C_T$ Method (Livak and Schmittgen, 2001). For each strain, at least two biological replicates were performed and at least two technical replicates were performed on each biological replicate.

In vitro Tissue Adherence Assay

An in vitro tissue adherence assay was performed as previously described (Magalhaes *et al.*, 2009). Briefly, paraffin-embedded C57BL/6 mice gastric sections were deparaffinized and rehydrated followed by blocking for two hours at room temperature with blocking buffer (1% periodate-oxidized BSA in PBS containing 0.05% Tween 20). FITC-labeled *H. pylori* was diluted to an OD600 of either 0.1, 0.05, 0.01, or 0.001 in blocking buffer and 100 μl of the bacterial suspension was then incubated with the tissue section for two hours at room temperature. Inhibition of SabA-mediated bacterial adherence was tested by addition of 1 ng μl-1 sialyl-Lewis[x] (sLex, Dextra) to the bacterial suspension immediately prior to incubation with the tissue section. Slides were subject to three ten minute washes in PBS containing 0.05% Tween 20 with gentle shaking and stained with DAPI. Mean number of adhered bacteria per square micrometer of tissue was quantified by analyzing at least five different fields of each section under 400X magnification using the ImageJ software. For each strain and condition, at least two biological replicates were performed. Statistical significance of differences in number of adherent bacteria per square micrometer was tested using the Student's t-test.

ELISA-based Adherence Assay

An ELISA assay to assess *H. pylori* binding to sLex was performed as previously described (Solnick *et al.*, 2004). Briefly, Immulon 2HB 96 well plates (Thermo Scientific) were coated with immobilized sLex conjugated to human serum albumin and incubated with digoxigenin-labeled *H. pylori*. Adherent *H. pylori* were detected using anti-digoxigenin antibody conjugated to peroxidase and ABTS (Roche). Absorbance was measured using an EL808 microplate reader (Bio-Tek Instruments) at a wavelength of 405 nm and normalized to uncoated control wells. To examine the effect of competitive binding with free sLex,

either 1 or 10 ng μl^{-1} sLex was added to the *H. pylori* media immediately prior to incubation in the sLex-coated 96 well plate. Percent adherence relative to adding no free sLex was calculated by first normalizing the sLex coated wells to the uncoated wells (sLex coated/uncoated) and then subtracting 1 to adjust the range of values so that a non-binding strain would have a value of 0. For each strain, at least two biological and technical replicates were performed.

Acknowledgments

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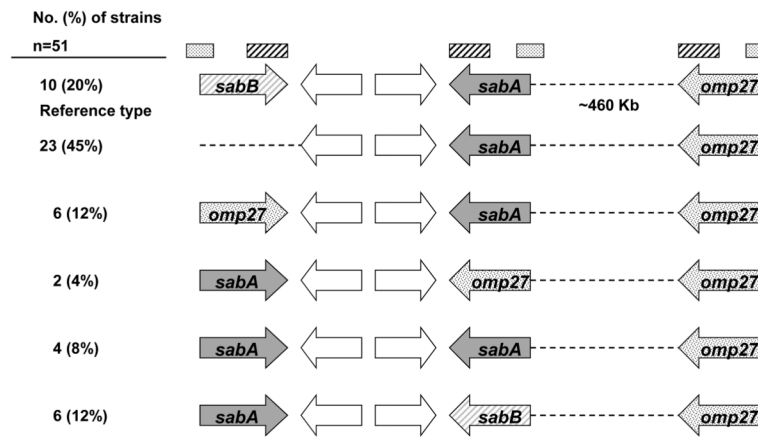
Figure 1.
Configuration of the *sabB* (*jhp0659*), *sabA* (*jhp0662*), and *omp27* (*jhp1103*) genes in the sequenced *H. pylori* reference strain J99 and *H. pylori* strain NSH57 and 51 clinical *H. pylori* strains. J99 and NSH57 share the same configuration denoted as reference type. Dotted and striped bars at the top represent the regions of homology (80 – 100% nucleotide identity) among these three *omp* genes that may direct recombination events.
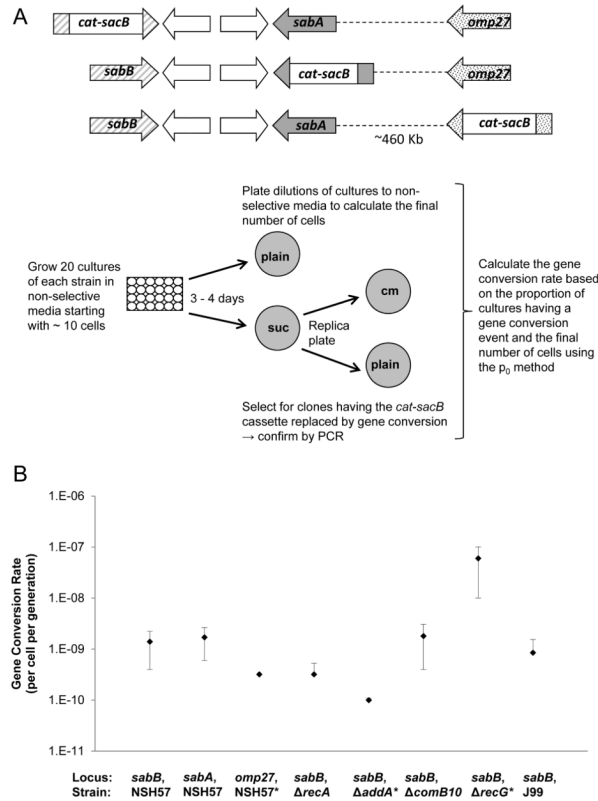
Figure 2.
Gene conversion rates and 95% confidence intervals as determined by fluctuation analysis. A) Experimental methods used to conduct fluctuation analysis. *H. pylori* strains were engineered with a *cat-sacB* cassette in place of the unique middle region of either the *sabB*, *sabA*, or *omp27* gene. The 5′ and 3′ homologous ends were left to allow recombination to occur. Twenty cultures of the engineered strain were grown from ~10 cells in non-selective media for ~24 generations. At the end of the incubation period, dilutions of the cultures were plated onto non-selective (plain) plates to determine the final number of cells in the culture. Clones having a spontaneous gene conversion will be sucrose-resistant and chloramphenicol-sensitive due to replacement of the *cat-sacB* cassette by an *omp* gene. Clones having a gene conversion were identified by selection on media containing sucrose (suc) followed by replica plating onto non-selective media and media containing chloramphenicol (cm). PCR was used to confirm replacement of the *cat-sacB* cassette with a duplicated copy of an *omp* gene. Gene conversion rates were calculated using the p_0 method (Foster, 2006). B) Gene conversion rates at the *sabB* locus (duplication of *sabA*, loss of *sabB*), the *sabA* locus (duplication of *sabB*, loss of *sabA*), and the *omp27* locus in *H. pylori* strain NSH57 and at the *sabB* locus in a *recA* deletion mutant, an *addA* deletion mutant, a *comB10* deletion mutant, a *recG* deletion mutant, and *H. pylori* strain J99. Gene conversion was not observed at the *omp27* locus and the lower limit of detection for the assay was 3.2×10^{-10} gene conversions per cell per generation. Asterisk indicates that the 95% confidence interval does not overlap with the 95% confidence interval for the gene conversion rate at the *sabB* locus in the NSH57 background.
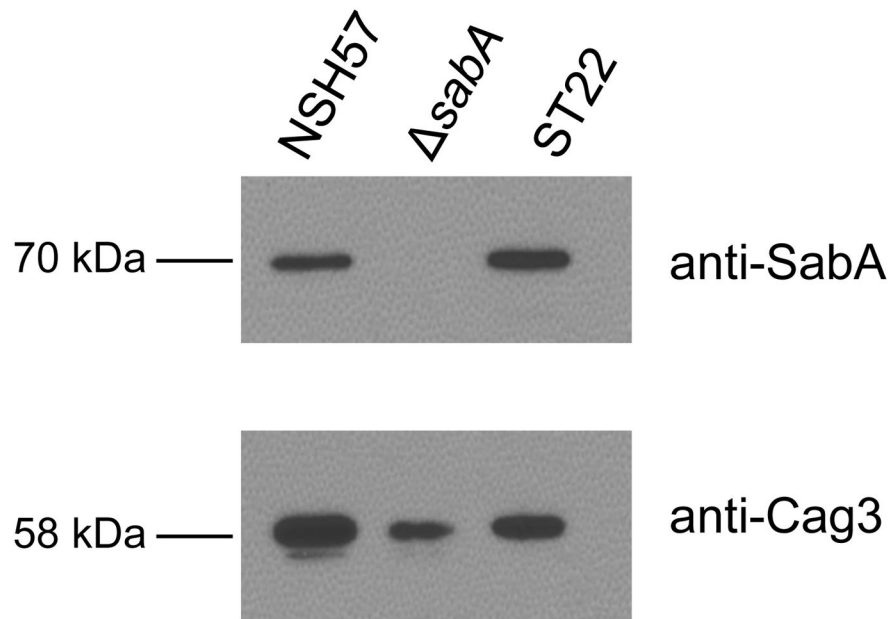
Figure 3.
Immunoblot analysis of SabA protein production of NSH57 having one *sabA* gene copy, a *sabA* deletion mutant, and ST22, a variant clone of NSH57 having two *sabA* gene copies with the second copy at the *sabB* locus. The relative amount of SabA protein produced was adjusted to the total protein loaded by stripping the blot and re-probing for Cag3 protein.
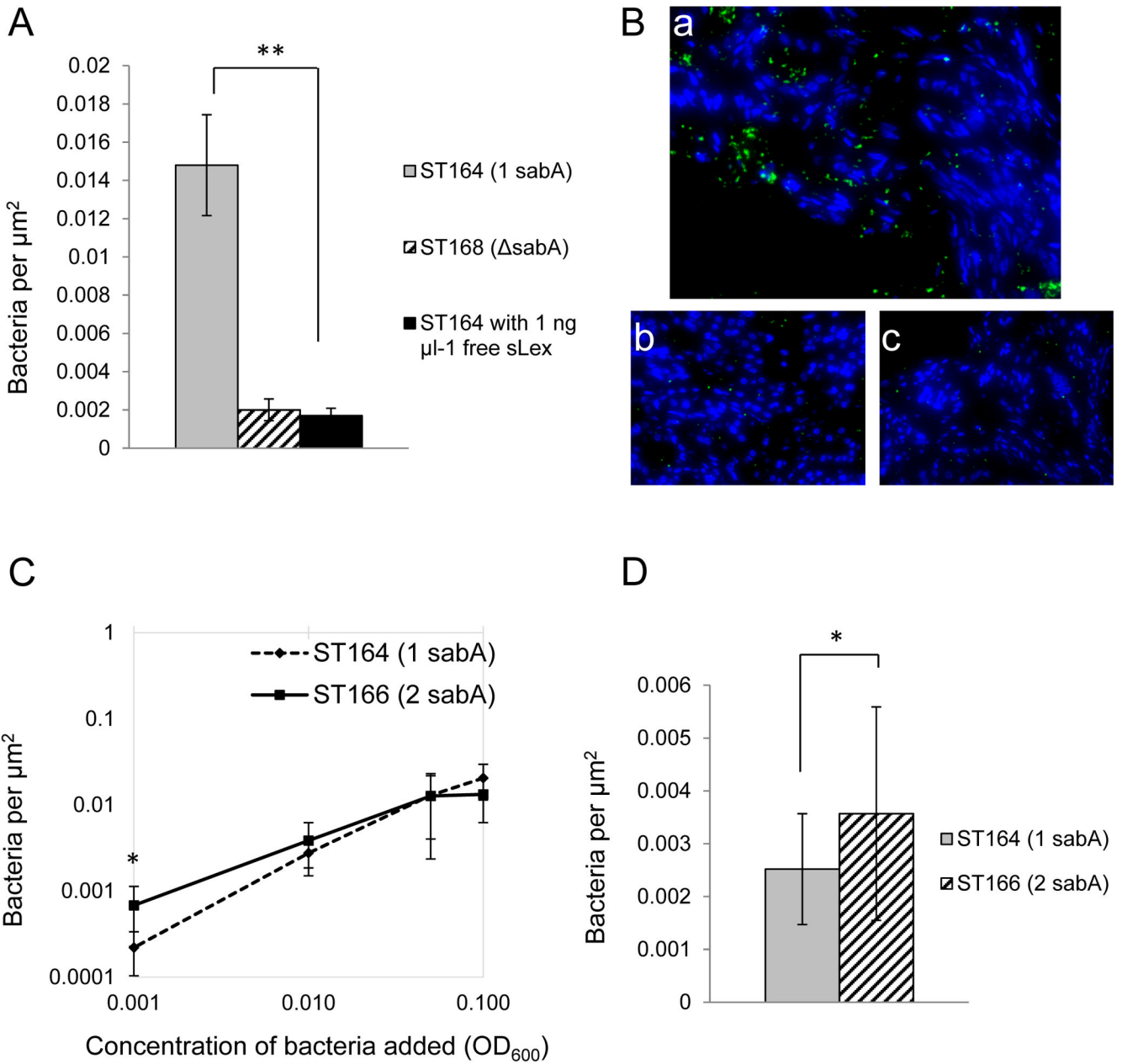
Figure 4.
Comparison of adherence to mouse gastric tissue of *H. pylori* strains ST164 (1 copy *sabA*, Δ*babA*), ST166 (2 copy *sabA*, Δ*babA*), and ST168 (Δ*sabA*, Δ*babA*). A–B) Adherence of ST164 (a), ST168 (b), and ST164 with 1 ng μl-1 free sLex added immediately prior to incubation with the mouse gastric tissue (c). The concentration of *H. pylori* added to the tissue was OD_{600} 0.1. The mean number adherent bacteria per μm^2 was calculated from 8 different fields for ST164 and from 5 different fields for ST168 and ST164 with 1 ng μl-1 free sLex. Results are representative of three independent experiments. Magnification is 400x. C) Quantification of adherence of ST164 and ST166 at different concentrations (OD_{600} 0.1, 0.05, 0.01, 0.001) of *H. pylori* incubated with the mouse gastric tissue. The mean number of adherent bacteria per μm^2 was calculated by combining data from two independent experiments. D) Quantification of adherence of ST164 and ST166. The mouse

gastric tissue was incubated with *H. pylori* at a concentration of OD_{600} 0.01. The mean number of adherent bacteria per μm^2 was calculated by combining data from five independent experiments. Statistical significance was evaluated using the Student's t-test. $p < 0.0001$ (**) and $p < 0.01$ (*).
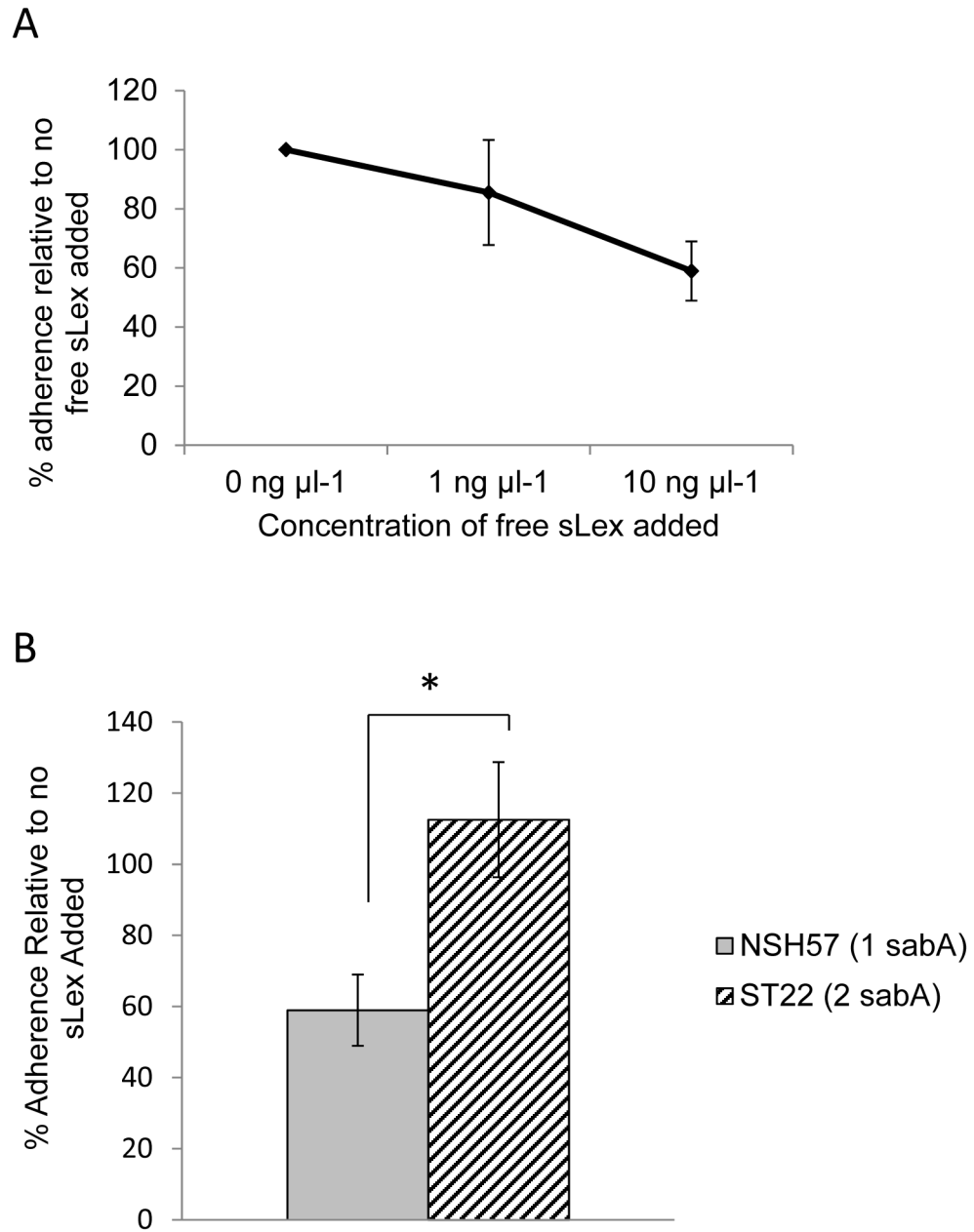
Figure 5.
SabA adherence to sialyl-Lewis^x (sLex) of wild-type *H. pylori* strain NSH57 having one *sabA* gene copy and ST22, a variant clone of NSH57 having two *sabA* gene copies with the second copy at the *sabB* locus. A) Inhibition of NSH57 adherence to immobilized sLex by addition of 1 ng μl^{-1} or 10 ng μl^{-1} free sLex to the *H. pylori* medium. B) Inhibition of NSH57 and ST22 adherence to immobilized sLex by addition of 10 ng μl^{-1} free sLex to the *H. pylori* medium. Percent adherence relative to adding no free sLex was calculated by first normalizing the sLex coated wells to the uncoated wells (sLex coated/uncoated) and then subtracting 1 to adjust the range of values so that a non-binding strain would have a value of 0. Mean ± standard deviation is calculated from three (NSH57 plus 1 ng μl^{-1} free sLex, ST22 plus 10 ng μl^{-1} free sLex) and four (NSH57 plus 10 ng μl^{-1} sLex) independent

experiments. Statistical significance of difference in inhibition of adherence was evaluated using the Student's t-test. P < 0.01 (*)

Table 1

H. pylori strains used in this study

Name	Genotype
NSH57	wild-type *H. pylori*
ST1	Δ*sabB::cat-sacB* in NSH57
ST2	Δ*sabA::cat-sacB* in NSH57
ST3	Δ*omp27::cat-sacB* in NSH57
ST27	Δ*comB10::aphA3* in ST1
ST40	Δ*recG::aphA3* in ST1
ST42	Δ*recA::aphA3* in ST1
SWH1	Δ*addA::aphA3* in ST1
ST4	Δ*sabB::sabA* in NSH57
ST22	Δ*sabB::sabA* with *sabA* promoter in NSH57
J99	wild-type *H. pylori*
ST73	Δ*sabB::cat-sacB* in J99
ST83	Δ*sabB::sabA* in J99
ST100	Δ*sabB::sabA* in J99
ST101	Δ*sabB::sabA* with *sabA* promoter in J99
ST164	Δ*babA::cat* in NSH57
ST166	Δ*babA::cat* in ST22
ST168	Δ*babA::cat*, Δ*sabA* in NSH57

Table 2

Oligonucleotides used in this study

Primer	Sequence	Position
jhp0658F	tgggttgagatcatgcaagcat	nt 323 to 344 of J99 jhp0658
jhp0660R	gatcatgcgtttttgatccctgg	nt 1249 to 1271 of J99 jhp0660 (*dcuA*)
jhp0661F	agggtgcttttacaactcgct	nt 925 to 945 of J99 jhp0661 (*ansB*)
jhp0663R	taggcaaacgcaaccgcttcaa	nt 746 to 767 of J99 jhp0663
jhp1102F	gttaaacccgctctaaactcggtg	nt 42 to 65 of J99 jhp1102
jhp1104R	gttatacgccacggcgatggaa	nt 552 to 573 of J99 jhp1104 (*deoD*)
sabB	ctgataaggcacagagattgcct	nt 526 to 548 of J99 jhp0659 (*sabB*)
sabA	aacaccgcgtattgcgttgggta	nt 749 to 771 of J99 jhp0662 (*sabA*)
omp27TypeI	tgccattctcatcggtgtagtg	nt 415 to 436 of J99 jhp1103 (*omp27* allele Type I)
omp27TypeII	gttttaatggttacttccacc	nt 606 to 626 of Tx30a *omp27* gene (allele Type II)
sabBFor	aagctcaaggcaatctctgtgc	nt 521 to 542 of HPG27_677 (*sabB*)
sabBRev	ccgcatagtcggttacaggaccag	nt 716 to 739 of HPG27_677 (*sabB*)
sabA_NSH57_RTFor	ctagaggcttgtttaccactgc	nt 389 to 410 of HPG27_680 (*sabA*)
sabA_J99_RTFor	aagtgagatagttccccgcat	nt 357 to 377 of J99 jhp0662 (*sabA*)
sabA_RTRev	acgccaacaacattgagctggt	nt 215 to 236 of HPG27_680 (*sabA*)
JHP0298For	gcgataaaacgccagatccaaagc	nt 845 to 868 of J99 jhp0298
JHP0298Rev	ctgggtattgcggtgctaatggg	nt 1001 to 1023 of J99 jhp0298

Table 3

Gene conversion rates and 95% confidence limits as determined by fluctuation analysis using the p$_0$ method

Locus	Strain background	Experiment	Proportion of cultures having gene conversion	Final no. cells	Gene Conversion Rate (per cell per generation)	95% confidence limits	
sabB	wild-type, NSH57	1	5 of 16	1.0×10^8	3.8×10^{-9}	8.9×10^{-10}	7.8×10^{-9}
sabB	wild-type, NSH57	2	4 of 17	3.6×10^8	7.5×10^{-10}	9.5×10^{-11}	1.6×10^{-9}
sabB	**wild-type, NSH57**	**combined**	**9 of 33**	$\mathbf{2.3 \times 10^8}$	$\mathbf{1.4 \times 10^{-9}}$	$\mathbf{5.6 \times 10^{-10}}$	$\mathbf{2.4 \times 10^{-9}}$
sabA	wild-type, NSH57	1	7 of 18	2.3×10^8	2.1×10^{-9}	7.8×10^{-10}	4.1×10^{-9}
sabA	wild-type, NSH57	2	4 of 19	1.9×10^8	1.2×10^{-9}	1.5×10^{-10}	2.6×10^{-9}
sabA	**wild-type, NSH57**	**combined**	**11 of 37**	$\mathbf{2.1 \times 10^8}$	$\mathbf{1.7 \times 10^{-9}}$	$\mathbf{7.7 \times 10^{-10}}$	$\mathbf{2.8 \times 10^{-9}}$
omp27	wild-type, NSH57	1	0 of 10	1.0×10^8	$< 1.1 \times 10^{-9}$		
omp27	wild-type, NSH57	2	0 of 19	1.1×10^8	$< 4.9 \times 10^{-10}$		
omp27	**wild-type, NSH57**	**combined**	**0 of 29**	$\mathbf{1.1 \times 10^8}$	$\mathbf{< 3.2 \times 10^{-10}}$ *		
sabB	ΔcomB10, NSH57	1	4 of 19	6.3×10^7	3.8×10^{-9}	4.4×10^{-10}	8.0×10^{-9}
sabB	ΔcomB10, NSH57	2	3 of 20	1.5×10^8	1.1×10^{-9}	4.3×10^{-11}	2.4×10^{-9}
sabB	**ΔcomB10, NSH57**	**combined**	**7 of 39**	$\mathbf{1.1 \times 10^8}$	$\mathbf{1.8 \times 10^{-9}}$	$\mathbf{5.5 \times 10^{-10}}$	$\mathbf{3.2 \times 10^{-9}}$
sabB	ΔrecA, NSH57	1	0 of 19	8.0×10^7	$< 6.8 \times 10^{-10}$		
sabB	ΔrecA, NSH57	2	2 of 20	1.3×10^8	8.1×10^{-10}	2.4×10^{-10}	2.0×10^{-9}
sabB	ΔrecA, NSH57	3	0 of 19	1.1×10^8	$< 4.9 \times 10^{-10}$		
sabB	**ΔrecA, NSH57**	**combined**	**2 of 58**	$\mathbf{1.1 \times 10^8}$	$\mathbf{3.2 \times 10^{-10}}$	$\mathbf{1.1 \times 10^{-10}}$	$\mathbf{7.7 \times 10^{-10}}$
sabB	ΔrecG, NSH57	1	2 of 10	1.9×10^6	1.2×10^{-7}	2.5×10^{-8}	3.1×10^{-7}
sabB	ΔrecG, NSH57	2	5 of 20	5.6×10^6	5.1×10^{-8}	1.1×10^{-8}	1.0×10^{-7}
sabB	**ΔrecG, NSH57**	**combined**	**7 of 30**	$\mathbf{4.4 \times 10^6}$	$\mathbf{6.0 \times 10^{-8}}$ *	$\mathbf{1.9 \times 10^{-8}}$	$\mathbf{1.1 \times 10^{-7}}$
sabB	ΔaddA, NSH57	1	0 of 10	3.9×10^8	$< 2.7 \times 10^{-10}$		
sabB	ΔaddA, NSH57	2	1 of 13	4.6×10^8	1.7×10^{-10}	1.4×10^{-10}	5.5×10^{-10}
sabB	**ΔaddA, NSH57**	**combined**	**1 of 23**	$\mathbf{4.3 \times 10^8}$	$\mathbf{1.0 \times 10^{-10}}$ *	$\mathbf{9.1 \times 10^{-11}}$	$\mathbf{3.2 \times 10^{-10}}$
sabB	wild-type, J99	1	3 of 18	1.5×10^8	1.2×10^{-9}	3.7×10^{-11}	2.8×10^{-9}
sabB	wild-type, J99	2	2 of 19	1.9×10^8	5.9×10^{-10}	1.7×10^{-10}	1.5×10^{-9}
sabB	**wild-type, J99**	**combined**	**5 of 37**	$\mathbf{1.7 \times 10^8}$	$\mathbf{8.5 \times 10^{-10}}$	$\mathbf{1.5 \times 10^{-10}}$	$\mathbf{1.7 \times 10^{-9}}$

*
95% confidence interval does not overlap with the 95% confidence interval for the gene conversion rate at the sabB locus in the NSH57 background.