Title

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Journal

Journal of Bacteriology, 196(2)

ISSN

0021-9193

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Publication Date

2014-01-15

DOI

10.1128/jb.00633-13

Peer reviewed

JB
Journals.ASM.org

Environmental Determinants of Transformation Efficiency in *Helicobacter pylori*

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Helicobacter pylori **uses natural competence and homologous recombination to adapt to the dynamic environment of the stomach mucosa and maintain chronic colonization. Although *H. pylori* competence is constitutive, its rate of transformation is variable, and little is known about factors that influence it. To examine this, we first determined the transformation efficiency of *H. pylori* strains under low O_2 (5% O_2, 7.6% CO_2, 7.6% H_2) and high O_2 (15% O_2, 2.9% CO_2, 2.9% H_2) conditions using DNA containing an antibiotic resistance marker. *H. pylori* transformation efficiency was 6- to 32-fold greater under high O_2 tension, which was robust across different *H. pylori* strains, genetic loci, and bacterial growth phases. Since changing the O_2 concentration for these initial experiments also changed the concentrations of CO_2 and H_2, transformations were repeated under conditions where O_2, CO_2, and H_2 were each varied individually. The results showed that the increase in transformation efficiency under high O_2 was largely due to a decrease in CO_2. An increase in pH similar to that caused by low CO_2 was also sufficient to increase transformation efficiency. These results have implications for the physiology of *H. pylori* in the gastric environment, and they provide optimized conditions for the laboratory construction of *H. pylori* mutants using natural transformation.**

H. pylori is a naturally competent (1), Gram-negative bacterium that colonizes the human gastric mucosa and causes peptic ulcer or gastric cancer in about 10% of those infected (2). Strains isolated from different individuals demonstrate extraordinary genomic diversity (3). Like other organisms with small genomes (4), *H. pylori* has relatively few regulatory networks (5) and instead exploits natural competence and genetic changes to adapt to changing conditions (6). Since infection is chronic over the lifetime of the host, sometimes with more than one resident strain (7–9), frequent genetic exchange enables *H. pylori* to better adapt to the changing environment of the host stomach (10, 11) and to acquire antibiotic resistance (12). Thus, natural competence is essential to the lifestyle of *H. pylori*.

H. pylori natural competence is mediated by a two-step process: uptake of double-stranded DNA across the outer membrane followed by transport of single-stranded DNA through the inner membrane (13). DNA is transported across the *H. pylori* outer membrane and into the periplasm via a ComB type IV secretion system (T4SS) similar to the Vir system of *Agrobacterium tumefaciens*. ComB components are expressed from two operons and have been named according to homology with Vir proteins. Several have been shown to be essential for *H. pylori* natural competence. For example, ComB9, homologous to VirB9, is thought to be an important structural component, tightly anchored to the outer membrane within the periplasmic space (1), and ComB4 is an ATPase putatively responsible for function of the ComB structure (13). Both proteins are thought to be involved in the biogenesis of the ComB apparatus (1). Once inside the periplasm, double-stranded DNA is processed to single-stranded DNA and transported across the bacterial inner membrane by a ComEC channel (14). Single-stranded DNA can then become incorporated into the bacterial chromosome via RecA-mediated homologous recombination (13).

Transformation efficiency in *H. pylori* is variable and can be affected by the length and similarity of DNA sequences and restriction-modification systems (15–17). Variation also occurs during *H. pylori* growth phases, with peaks at mid-log- and late-stationary-phase growth (18). Since natural transformation is an important mechanism for bacterial adaptation to the gastric environment, certain conditions of stress may increase *H. pylori* competence. For example, induction of double-strand breaks by treatment with ciprofloxacin can increase expression of *comB* genes and enhance competence (19). Taken together, these studies suggest that *H. pylori* has evolved to increase natural competence when this is beneficial for its survival.

Another stress condition that might alter *H. pylori* competence is a change in the partial pressure of O_2 or CO_2. CO_2 is highly prevalent in the gastric environment, while O_2 tension is low. In the live mouse, for example, the partial O_2 pressure (pO_2) in the stomach is estimated at 58 ± 15 torr (~7.6%) (20). O_2 and CO_2 concentrations are variable within the gastric environment and can be affected by disease state, food consumption, and location within the gastric mucosa (21, 22). For example, the CO_2 concentration tends to be low nearest the epithelium (where the pH is high) and higher toward the gastric lumen (where the pH is low). Although, *H. pylori* is generally regarded as a microaerophile (2), it expresses high levels of catalase and superoxide dismutase, which facilitate the elimination of toxic oxygen intermediates (23, 24). More recent studies have shown that many strains can tolerate and even thrive in the presence of high O_2 levels as long as CO_2 levels are high (25). These studies indicate that *H. pylori* is a capnophile whose growth is promoted by atmospheric O_2 as long as sufficient CO_2 is present.

Here we show that low levels of CO_2 markedly increase *H.*

Received 29 May 2013 **Accepted** 28 October 2013
Published ahead of print 1 November 2013
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JB.00633-13.

doi:10.1128/JB.00633-13

TABLE 1 Transformation conditions and defined gas mixtures

Transformation conditions (%, vol/vol)			Defined gas mixture (%, vol/vol)		
Oxygen	Carbon dioxide	Hydrogen	Carbon dioxide	Hydrogen	Nitrogen
5.0	7.6	7.6	10.0	10.0	80.0
15.0	2.9	2.9	10.0	10.0	80.0
15.0	7.7	7.7	27.0	27.0	46.0
5.0	2.9	7.6	3.8	10.0	86.2
5.0	7.6	2.9	10.0	3.8	86.2

pylori transformation efficiency and induce expression of the ComB T4SS. This change in competence seems to be a result of CO_2 influence on the pH of the bacterial culture medium. These results are important for understanding the physiology of *H. pylori* in the gastric environment and also have practical implications for using natural transformation to generate *H. pylori* mutants in the laboratory.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Helicobacter pylori* strains J166 and SS1 were cultured on brucella agar (Becton, Dickinson and Company, Sparks, MD) supplemented with 5% heat-inactivated newborn calf serum (Gibco, Grand Island, NY), 5 μg/ml trimethoprim, 10 μg/ml vancomycin, 2.5 units/ml polymyxin B, and 2.5 μg/ml amphotericin B (Sigma-Aldrich, Inc., St. Louis, MO). Cultures were grown at 37°C using the Anoxomat system (Advanced Instruments, Inc., Norwood, MA), which is a sealed jar connected to a pressurized tank that contained one of several defined gas mixtures (Table 1). Specific gas conditions in the sealed jar were achieved by removing a defined volume of ambient air and replacing it with the appropriate defined gas. Transformation efficiency was initially studied under 5% O_2 (7.6% CO_2, 7.6 H_2) or 15% O_2 (2.9% CO_2, 2.9% H_2), where O_2 was manipulated by changing the volume of a single defined gas mixture, allowing the other gases to vary. Separate defined gas mixtures were then used to manipulate O_2, CO_2, and H_2 individually, using N_2 as a replacement gas (Table 1).

Plasmid construction. Two plasmids were used to measure transformation efficiency, one that deleted an outer membrane protein (pOMP) and a second that deleted an intergenic region (pIG) between oppositely oriented genes and did not disrupt a known open reading frame. A third plasmid (pRecA) was developed to disrupt the *recA* gene to determine the influence of RecA expression on natural competence genes under relevant experimental conditions. Construction of the plasmids is shown schematically in Fig. 1; primer sequences are given in Table 2. To construct pOMP for strain J166 (pOMPJ166), a chloramphenicol resistance cassette (26) was amplified and ligated to DNA sequences from upstream and downstream of the promoter region in the *babA* gene (designated HP1243 in *H. pylori* 26695), which encodes the blood group antigen binding protein (27). A similar plasmid was constructed for strain SS1 (pOMPSS1), except that upstream and downstream sequences were amplified so as to delete the entire HP1243 locus, which in SS1 carries a *babA* paralog, *babC*. To construct pIG, a nonpolar kanamycin cassette (aphA3) was amplified from pUC18K (28) and ligated between HP0512 and HP0519 amplified from strain J166 (pIGJ166) or SS1 (pIGSS1), which in both strains are transcribed in opposite directions and contain no intervening open reading frames. These features, along with the insertion of a nonpolar cassette, minimized potential effects on the strain phenotype. Construction of pRecA was the same as for pIG except that aphA3 was ligated into the *recA* open reading frame of *H. pylori* SS1. PCR-amplified fragments were digested with the appropriate enzymes (New England BioLabs, Ipswich, MA), ligated into NotI/XhoI-digested pBluescript SK− vector (Stratagene, La Jolla, CA) using T4 DNA ligase (New England BioLabs, Ipswich, MA) according to the manufacturer's instructions, and cloned into *Escherichia coli* Top10 (Invitrogen, Carlsbad CA) with selection on chloramphenicol (5 μg/liter) or kanamycin (25 μg/liter) as appropriate.

Transformations. Transformations were performed as previously described (29). Briefly, overnight (18- to 24-h) *H. pylori* cultures were harvested from agar plates using a sterile swab and suspended in brucella broth to an optical density at 600 nm (OD_{600}) of 5, and 10 μl was spread evenly in a 1.5-cm-diameter circle on a fresh agar plate. Five replicate circles of these bacterial cells were then incubated at 37°C in an Anoxomat jar adjusted to contain the appropriate atmospheric conditions. After 3 to 6 h, 0.3 to 3 μg of plasmid or genomic DNA suspended in deionized water was mixed with each of the circles using a sterile loop. The cells with DNA were then returned to 37°C in the same gas mixture with which they had been conditioned. After overnight incubation, the bacteria were removed from the 1.5-cm circle using a sterile loop and suspended in 300 μl brucella broth. Dilutions were plated on nonselective and selective plates with 5 μg/ml chloramphenicol or 25 μg/ml kanamycin. Transformation efficiency was calculated as the number of CFU present on selective medium divided by the number of CFU on nonselective medium divided by the amount (μg) of DNA used for transformation.

Effects of growth phase on competence. *H. pylori* SS1 harvested from an overnight agar culture was plated on brucella agar in 1.5-cm-diameter circles as described for transformations. Plates were incubated under 5% or 15% O_2 conditions, and at 6-h intervals from 0 to 30 h, 3.0 μg of pIGSS1 was added to three replicate circles and mixed with a sterile loop. After 6 h of additional incubation with DNA, bacteria were removed from the circles with a sterile loop, suspended in brucella broth, and then plated and analyzed for both CFU and transformation efficiency.

RNA extraction and quantitative reverse transcription-PCR (qRT-PCR). A 100-μl suspension in brucella broth (OD_{600} = 5) was harvested from an overnight brucella agar culture of *H. pylori* SS1, spread evenly on a brucella agar plate, and incubated at 37°C with 5% or 15% O_2 for 18 to 24 h. Bacterial cells were suspended in 1 ml TRIzol reagent (Ambion,

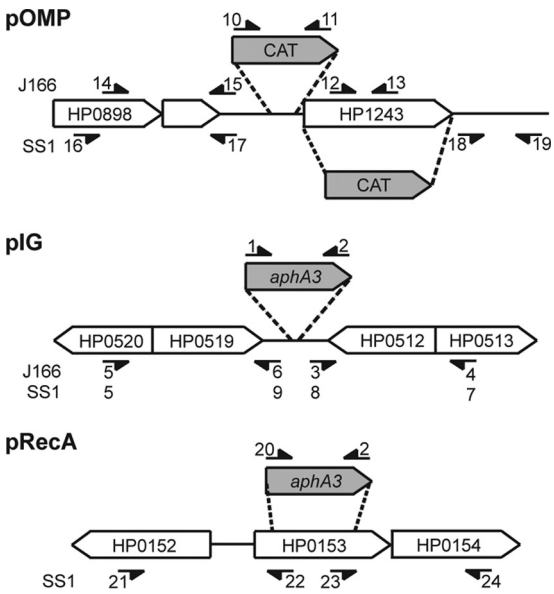

FIG 1 Schematic of plasmid construction. pOMP was constructed by inserting a chloramphenicol resistance cassette (CAT) into the promoter region (*H. pylori* J166) or the open reading frame of *babA* (HP1234) (*H. pylori* SS1). pIG was constructed by insertion of a kanamycin resistance cassette (aphA3) into an intergenic region between HP0519 and HP0512 in *H. pylori* J166 and SS1. pRecA was constructed by insertion of aphA3 into the *recA* gene in *H. pylori* SS1. The primers used for amplification of plasmid components for each strain are represented by small numbered arrows and correspond to the primers in Table 2. Gene designations are for *H. pylori* 26695.

TABLE 2 Primers used for plasmid construction and RT-PCR

Number	Primer name	Restriction site	Sequence (5′ → 3′)[a]
1	aphA3_F	SacI	AAC GAGCTC GGT ACC CGG GTG AC
2	aphA3_R	HincII	AAC GTCGAC TCT AGA GGA TCC CC
3	J166_IG_F1	HincII	AAT GTCGAC AAA TCT TAT TAT CGA CAC TCT ATT AGA A
4	J166_IG_R1	XhoI	AAT CTCGAG CCC TTT GTT GGC CGT CAA
5	J166_IG_F2	NotI	AAT GCGGCCGC TAG CAC CGC TCA AGA CAC AA
6	J166_IG_R2	SacI	AAT GAGCTC CCA AGT GAT AGT AAA GAT TAG GTA A
7	SS1_IG_F1	XhoI	AAT CTCGAG ATC ATA CCC CTA TTT CTA GGA
8	SS1_IG_R1	HincII	AAT GTCGAC AAA TCT TAT CAT CGA CAC TCT ATT AAA G
9	SS1_IG_R2	SacI	AAT GAGCTC TCA AGT GAT AGT AAA GAT TAG GTA G
10	cat_F	SacI	AAC GAGCTC GAT GCT TTA TAA CTA TGG ATT AAA CAC
11	cat_R	BamHI	AAC GGATCC TTA TCA GTG CGA CAA ACT GGG AT
12	J166_OMP_F1	BamHI	AAC GGATCC TTG CTC CAC GCT GAA GAC
13	J166_OMP_R1	XhoI	AAC CTCGAG GAC GCT CGT TTG ATT GAC CA
14	J166_OMP_F2	NotI	AAC GCGGCCGC AGC CAC AAA ACC TCT AAA GA
15	J166_OMP_R2	SacI	AAC GAGCTC GGG GTA TTT TGA AAT AAC TCT C
16	SS1_OMP_F1	XhoI	AAC CTCGAG TTT TGA GCC GGT GGA TAT ATT AG
17	SS1_OMP_R1	SacI	AAC GAGCTC GCA AGC TCT TTT ATT ATT TAT CTT A
18	SS1_OMP_F2	BamHI	AAC GGATCC CGT TAA AAC CTT TTG TGA AAC T
19	SS1_OMP_R2	NotI	AAA GCGGCCGC TGT GGA TCT AGC GGT TCT T
20	aphA3_F2	PstI	AAT CTGCAG GGT ACC CGG GTG AC
21	SS1_RecA_F1	NotI	AAT GCGGCCGC TCG TTA CTG CCC TTA ATG AGC TC
22	SS1_RecA_R1	PstI	AAT CTGCAG CAA TTT GTT TGA TCG CTA AAG AAA TCG C
23	SS1_RecA_F2	HincII	AAT GTCGAC AAT GAA GAG ATC ATG CCC TTA CCC
24	SS1_RecA_R1	XhoI	AAT CTCGAG AAAAGACAATCAGGGAGCTATGGC
25	comB4_F1		TGT TAG AAA AGC TTT TAA GCG C
26	comB4_R1		TTG CTC TGT GCT TAA ATG GGT G
27	comB9_F		AAG CCG ATG ATT TTT TAG AAG AAG C
28	comB9_R		AAA GCT CCC TTG AAT GGC GTT TAA
29	recA_F		GTG CGC CTT GGG GAT AAG CA
30	recA_R		CTT AGA GTG GTC TTC CCG CTT GAC
31	16S_F		GGA GTA CGG TCG CAA GAT TAA A
32	16S_F		CTA GCG GAT TCT CTC AAT GTC AA

[a] Underlining indicates a restriction site.

Carlsbad, CA), and total RNA was isolated and then treated with DNase I (Roche Applied Science, Mannheim, Germany) according to the manufacturer's directions. RNA was purified using an RNeasy Minikit (Qiagen Sciences, Germantown, MD). Gene-specific primers (Table 2) were used to synthesize cDNA with the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. A 1-μl aliquot of a 1:5 dilution of cDNA was used to perform quantitative PCR with the same primers using SYBR green PCR master mix with standard cycling parameters (Applied Biosystems, Warrington, WA) in 20-μl reaction mixtures on an ABI 7900HT 96 well block.

Determination of CO_2 effects on brucella medium pH. The pH of 25 ml sterile brucella broth (Becton, Dickinson and Company, Sparks, MD) was measured using a pH meter (Beckman Coulter) after overnight incubation under 7.6% CO_2 (5% O_2) or 2.9% CO_2 (15% O_2) at 37°C. Brucella agar plates, supplemented with 11 mg/liter phenol red (Matheson, Coleman & Bell, Norwood, OH), were incubated under 7.6% CO_2 (5% O_2) or 2.9% CO_2 (15% O_2) at 37°C overnight. The pH of brucella agar plates from each condition was visually estimated by comparison to a standard curve, prepared by supplementing 25-ml aliquots of brucella broth with 11 mg/liter phenol red and adjusting the pH in increments of 0.2 from 5.8 to 6.8 using 6 N HCl or NaOH. The agar pHs of two to four brucella agar plates from each condition were independently estimated by four observers, and the average values are reported.

Statistical analysis. Pairwise comparisons were performed using the Student *t* test with Welch's correction on GraphPad Prism software. Data involving bacterial counts were log transformed prior to statistical analysis. Error bars in all figures represent standard error.

RESULTS

***H. pylori* transformation efficiency under 5% and 15% O_2.** To determine the transformation efficiency of *H. pylori* under different atmospheric conditions, we first incubated *H. pylori* SS1 with plasmid DNA directed to a *babA* paralog (pOMPSS1) and compared the results under 5% O_2 to 15% O_2 using a single defined gas mixture (Table 1). The transformation efficiency was increased approximately 6-fold in 15% O_2 (Fig. 2A). To determine if these results were strain specific, we repeated the experiment with *H. pylori* J166 using the corresponding plasmid, pOMPJ166. This resulted in approximately 8-fold-greater transformation efficiency in 15% O_2 than in 5% O_2 (Fig. 2B). The transformation efficiency could be increased under high O_2 conditions because deletion of an OMP increases *H. pylori* fitness under high O_2, though it seems unlikely since two different OMPs were targeted (*babA* in J166 and *babC* in SS1). In other words, the effect of O_2 on transformation efficiency could be specific to one genetic locus. To test this, we next transformed *H. pylori* SS1 and J166 with plasmid DNA containing a kanamycin resistance cassette directed to an intergenic region (pIGSS1 and pIGJ166) that would not be expected to disrupt an open reading frame. Under 15% O_2, the transformation efficiencies in strains SS1 and J166 increased 32-fold and 10-fold, respectively (Fig. 2C and D). These results show that a change in atmospheric conditions can cause a robust change

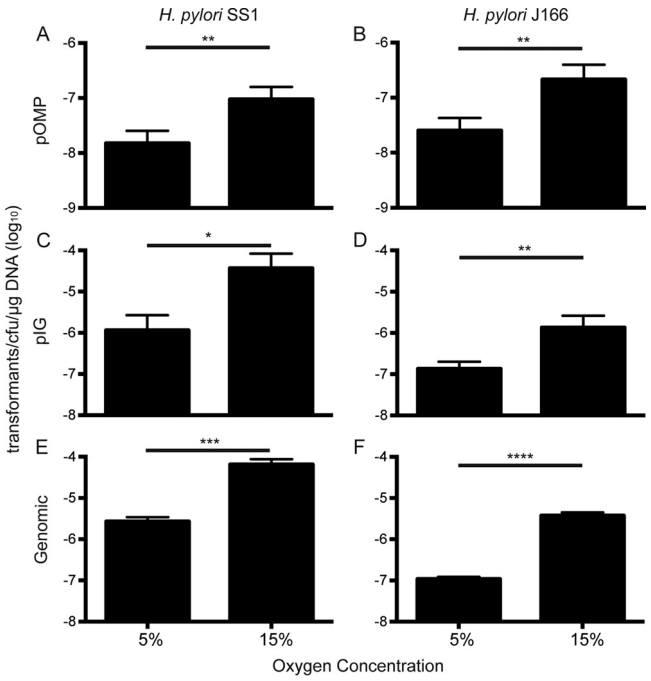

FIG 2 Transformation efficiency in *H. pylori* is increased under conditions of high O_2 and low CO_2. *H. pylori* SS1 and J166, as indicated, were transformed with pOMPSS1 (A), pOMPJ166 (B), pIGSS1 (C), pIGJ166 (D), genomic DNA from pIGSS1-transformed *H. pylori* SS1 (E) and genomic DNA from pIGJ166-transformed *H. pylori* J166 (F) at 5% O_2 (7.6% CO_2, 7.6% H_2) or 15% O_2 (2.9% CO_2, 2.9% H_2). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

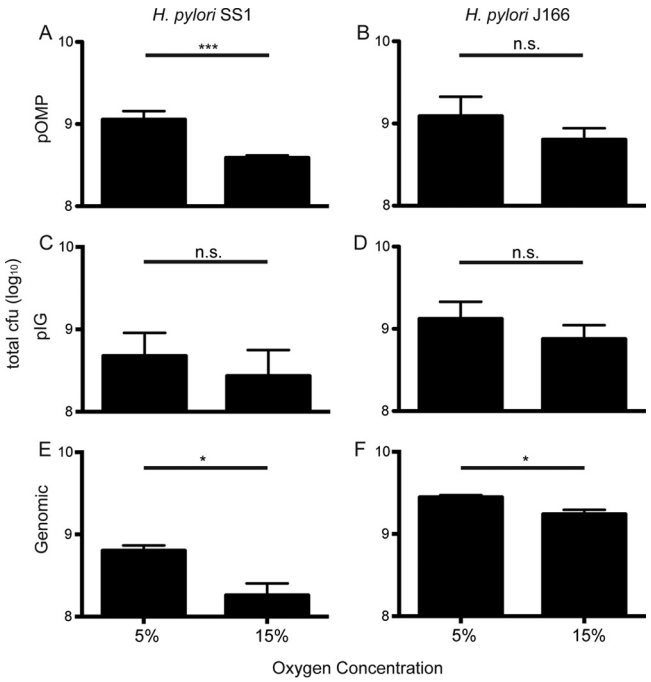

FIG 3 A trend of reduced *H. pylori* growth is observed under conditions of high O_2 and low CO_2. CFU for *H. pylori* SS1 (A, C, and E) and J166 (B, D, and F) under high and low O_2 conditions are shown. The data correspond to the transformation efficiency results shown in Fig. 2. *, $P < 0.05$; ***, $P < 0.001$; n.s., not significant.

in *H. pylori* natural competence that is independent of strain or genetic locus.

To ensure that this phenomenon was not specific to plasmid DNA, genomic DNA was isolated from *H. pylori* SS1 and J166 which had been transformed with pIGSS1 and pIGJ166, respectively. This DNA was then used to perform transformation efficiency experiments as described above. Under 15% O_2, the transformation efficiency increased 24-fold and 34-fold in strains SS1 and J166, respectively (Fig. 2E and F), illustrating that both plasmid and genomic DNAs are taken up by the bacteria with increased efficiency under 15% O_2 conditions.

Atmospheric effects on transformation efficiency are independent of growth phase. High O_2 conditions that increased transformation efficiency (Fig. 2) also resulted in a trend of decreased bacterial growth (Fig. 3). Since it has previously been shown that transformation efficiency is variable throughout *H. pylori* growth phases (18), we considered the possibility that the effects of changing atmospheric conditions on competence were due to altered growth. To address this, we measured the transformation efficiency of *H. pylori* SS1 at different growth phases under both 5% and 15% O_2. pIGSS1 DNA was added at 6-h intervals under 5% and 15% O_2, and the transformation efficiency was determined 6 h later. As expected, 15% O_2 caused a trend of decreased bacterial growth (Fig. 4A). No transformants were detected at 0 h, but when DNA was added after 6 to 30 h of growth (mid-log to stationary phase), the transformation efficiency was markedly higher at 15% O_2 at each point in the growth curve (Fig. 4B). Thus, although 15% O_2 moderately impaired *H. pylori* growth, it also increased competence at every point in the growth

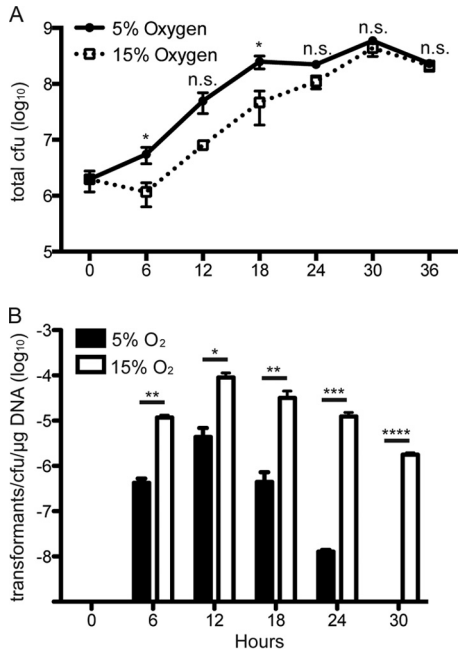

FIG 4 *H. pylori* growth and transformation efficiency are affected by atmospheric conditions throughout the growth curve. (A) Total CFU present under 5% O_2 (solid line) or 15% O_2 (dotted line) when transformation efficiency was measured for each time point shown. (B) Transformation efficiency of *H. pylori* SS1 under 5% O_2 (filled bars) or 15% O_2 (open bars), as measured 6 h after the addition of pIGSS1, which was added at the time points shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.001$, n.s. = not significant.

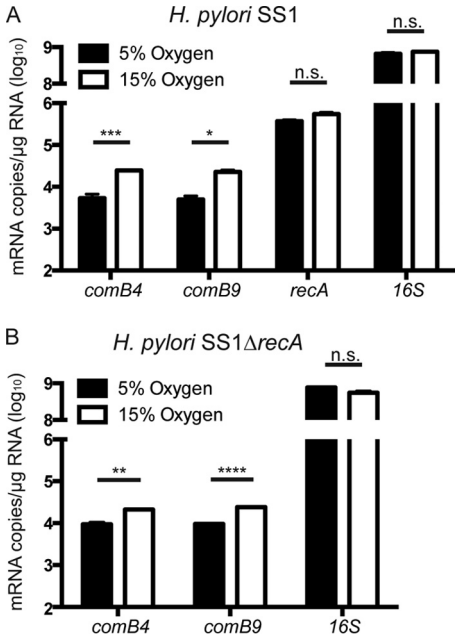

FIG 5 ComB expression is induced by conditions of high O_2 and low CO_2 independent of RecA. *H. pylori* SS1 (A) or SS1 Δ*recA* (B) mRNA expression levels of the *comB4*, *comB9*, *recA*, and 16S rRNA genes after overnight incubation with 5% O_2 (filled bars) or 15% O_2 (open bars) are shown. In both SS1 and SS1 Δ*recA*, *comB4* and *comB9* were significantly induced under 15% O_2. Induction of *recA* did not occur. 16S rRNA was used as a loading control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; n.s. = not significant.

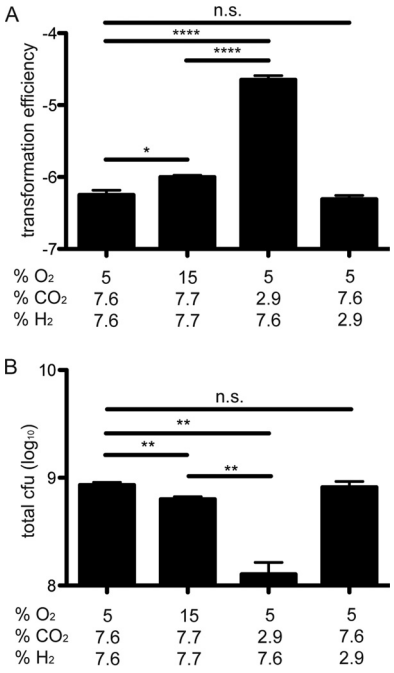

FIG 6 *H. pylori* transformation efficiency is induced by low CO_2 rather than high O_2 levels. Transformation efficiency as measured after the addition of pIGSS1 (A) and total CFU (B) of *H. pylori* SS1 under defined atmospheric conditions are shown. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$; n.s. = not significant.

curve, indicating that growth phase is not responsible for the increase in natural competence at 15% O_2 (Fig. 4).

The ComB T4SS is induced under 15% O_2 conditions independent of RecA. *H. pylori* natural competence occurs primarily through expression of the ComB T4SS apparatus. RecA is also an important protein involved in homologous recombination following uptake of exogenous DNA. Therefore, we examined expression of the *comB* and *recA* genes under both high and low O_2 conditions. Since the ComB apparatus is composed of elements present on two operons, *comB2-4* and *comB6-10* (30), we examined expression of one *comB* gene from each operon (*comB4* and *comB9*). qRT-PCR analysis of RNA isolated from *H. pylori* SS1 incubated under either high or low O_2 conditions showed significantly increased expression of both *comB4* and *comB9* under 15% O_2 conditions (Fig. 5A). No changes in expression of *recA* were seen. This result is in agreement with previous studies (19) indicating that *recA* expression was not increased during induction of other natural competence genes. 16S rRNA, which served as a loading control, was also unchanged. These results suggest that increased *H. pylori* competence under high O_2 is mediated by the ComB T4SS.

It has previously been shown that RecA is required for *H. pylori* induction of *comB* genes in response to DNA double-strand breaks (19). To determine whether the induction of *comB* genes in response to increased O_2 was also dependent on RecA, we developed a *recA* deletion mutant of *H. pylori* SS1 (SS1 Δ*recA*) using pRecA. qRT-PCR was performed on RNAs from three individual SS1 Δ*recA* clones incubated under either 5% or 15% O_2 conditions. Significant induction of both *comB4* and *comB9* was detected under 15% O_2 (Fig. 5B). These data indicate that induction of the ComB apparatus under 15% O_2 conditions occurs by a novel mechanism, independent of the response to double-strand break induction.

***H. pylori* natural competence is induced in response to low CO_2.** In all of the above-described experiments in which a single defined gas mixture was used, CO_2 and H_2 concentrations were decreased to accommodate an increased O_2 concentration. Because recent work shows that *H. pylori* is a capnophile (25, 31), we asked whether the change in CO_2, rather than O_2, was responsible for the increase in transformation efficiency. Transformation efficiency in *H. pylori* SS1 was examined using pIGSS1 under a series of conditions (Table 1) in which we individually manipulated O_2, H_2, or CO_2, leaving the other gases at the same levels found under low O_2 conditions. Nitrogen was used as a replacement gas. When O_2 was increased to 15% with no change in CO_2 or H_2, a small (1.8-fold) increase in competence was observed. In contrast, reducing CO_2 without changing O_2 or H_2 caused a marked (40-fold) increase in transformation efficiency (Fig. 6A). Reduction of H_2 alone had no effect. Additionally, the least amount of growth was measured under conditions of low CO_2, which resulted in the highest transformation efficiency (Fig. 6B). These results indicate that the increase in *H. pylori* transformation efficiency under different atmospheric conditions is predominantly a result of a decreased CO_2 concentration.

Increased pH mimics the increase in transformation efficiency caused by decreased CO_2. Since CO_2 reacts with water to form carbonic acid, differences in CO_2 concentration could affect the pH of the unbuffered culture medium. The pH of autoclaved brucella broth was 6.87 (±0.01), which decreased to 6.31 (±0.02) after overnight incubation under 7.6% CO_2 (5% O_2). Conditions

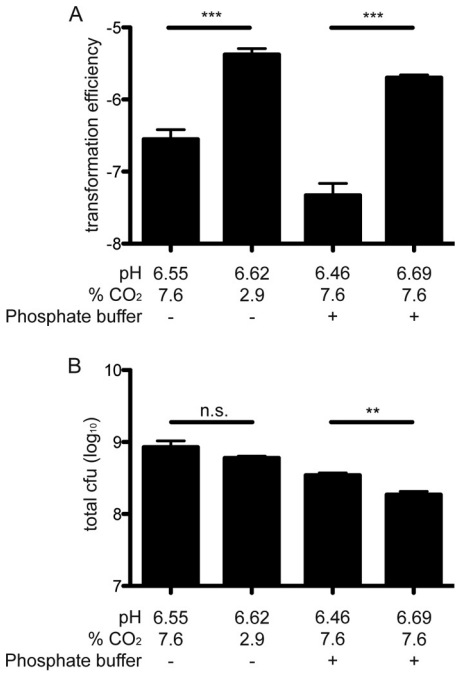

FIG 7 A change in pH is sufficient to reproduce the effects of CO_2 on *H. pylori* transformation efficiency. (A) The transformation efficiency of *H. pylori* SS1 was increased both by a decrease in CO_2 (2.9%) and by an increase in pH under buffered conditions (7.6% CO_2). (B) Increased transformation efficiency was accompanied by small decreases in CFU. **, $P < 0.01$; ***, $P < 0.001$; n.s. = not significant.

of low (2.9%) CO_2 resulted in a lesser decrease in pH, to 6.47 (±0.03), after overnight incubation. Since all transformations were done on agar-grown bacteria, phenol red was added to brucella agar medium prior to autoclaving in order to verify that a similar effect occurred in brucella agar plates. Brucella agar plates containing phenol red were incubated under 7.6% CO_2 or 2.9% CO_2 overnight. The color of the plates before and after incubation at each of the conditions was estimated by comparison to a phenol red standard curve. Under aerobic conditions, the pH of brucella agar was 6.76 (±0.04), and this decreased to 6.55 (±0.08) after incubation at 7.6% CO_2. Low (2.9%) CO_2 conditions again resulted in less of a decrease in pH, to 6.63 (±0.03). Thus, incubation under conditions of increased CO_2 caused a decrease in the pH of unbuffered brucella medium that was relative to the amount of CO_2 present.

To determine whether increased pH could be responsible for the increase in competence under low CO_2 conditions, transformation efficiency was determined on buffered brucella agar plates adjusted to pH 6.5 or 7.0 with 20 mM phosphate buffer. After incubation at 7.6% CO_2, the pH of the plates buffered at 6.5 or 7.0 decreased slightly to 6.46 (±0.06) and 6.69 (±0.02), respectively. *H. pylori* SS1 was transformed with pIGSS1 on these buffered plates under 7.6% CO_2. The transformation efficiency of *H. pylori* SS1 was about 35-fold higher with pIGSS1 at pH 6.69 than at pH 6.46 under 7.6% CO_2, which mimicked the increase in competence caused by conditions of low CO_2 and high O_2 (Fig. 7A). This was also accompanied by decreased growth (Fig. 7B). Incubation of *H. pylori* in liquid medium prepared in the same way as the plates described above showed that although the presence of *H. pylori* could moderately affect the absolute pH of the buffered medium, it did not significantly affect the difference in pH between the two buffers (see Fig. S1 in the supplemental material). Together these results show that increased pH is sufficient to increase transformation efficiency, and they suggest that this may explain the increased competence under conditions of low CO_2.

DISCUSSION

Genomic diversity is a signature feature of *H. pylori* that arises from mutation, recombination, and importation of exogenous DNA via natural transformation, which allows *H. pylori* to adapt to the hostile environment of the human stomach. Variability in competence among different *H. pylori* strains is well documented (16, 32, 33), but little is known about conditions that affect transformation efficiency. This is important, not only for understanding the physiology of *H. pylori* in the gastric environment but also for optimizing methods that are commonly exploited in the laboratory to generate isogenic mutants. Here we systematically examined the effects of environmental growth conditions on *H. pylori* transformation efficiency.

Competence in naturally transformable bacteria is commonly induced by physiological stress (34). Since *H. pylori* is considered a microaerophile that is subject to toxic oxygen species under ambient O_2 conditions (35), we first examined the impact of O_2 concentration on natural competence. Culturing *H. pylori* under 15% versus 5% O_2 decreased bacterial growth, increased transformation efficiency, and induced the *comB* operon, which is required for natural competence. These effects were robust across different *H. pylori* strains, genetic loci, and bacterial growth phases (Fig. 2 to 5). However, in these experiments manipulation of O_2 was accomplished using a single defined gas mixture, so the increase in O_2 was also accompanied by decreases in CO_2 and H_2. Since some studies have suggested that *H. pylori* is a capnophile (25, 31), we considered the possibility that low CO_2 rather than high O_2 was the relevant environmental variable responsible for increasing competence. Independent manipulation of O_2 and CO_2 revealed that in fact low CO_2 was primarily responsible for the increased transformation efficiency, though there was also a small effect of increased O_2, which can induce the *comB* operon (31). An increase in pH similar to that caused by incubating unbuffered agar plates under low CO_2 also increased transformation efficiency. Thus, increased pH is itself sufficient to enhance *H. pylori* competence. Increased pH has also been observed to enhance competence in *Campylobacter jejuni* (36, 37) and *Streptococcus pneumoniae* (38), which suggests that this response may be a convergent evolutionary adaptation of naturally competent, opportunistic mucosal pathogens.

It may at first seem paradoxical that *H. pylori* transformation efficiency increased under high pH (low CO_2), since the stomach is commonly thought of as a harsh, acidic environment in which the acidity might be expected to induce a stress response and increase competence. However, several lines of evidence are consistent with the observation that increased pH may induce stress in *H. pylori*. First, studies of gene expression *in vitro* and *in vivo* suggest that the natural habitat of *H. pylori* within the gastric mucosa is actually quite acidic, with a pH of ≤4.0 (39). In fact, *H. pylori* cannot survive neutral pH in the presence of physiological concentrations of urea because the pH rapidly rises (40). The stringent response is also required for *H. pylori* survival under high O_2 conditions (41), which can be abrogated by addition of

CO_2 (25). *Ex vivo* measurements of guinea pig gastric tissue indicate that a pH gradient exists across the mucosa, with a pH of approximately 6.1 immediately adjacent to the epithelial cell surface that gradually decreases toward the gastric lumen (42) Therefore, *in vivo* it is likely that increased pH and reduced CO_2 would be experienced immediately adjacent to the host epithelial cells (43), while *H. pylori* is thought to more commonly localize just offshore in the mucous layer (44). It may be that *H. pylori* undergoes a stress response when located close to the gastric epithelium, with consequent upregulation of ComB and increased competence.

We conclude that the increased transformation efficiency observed *in vitro* under elevated pH and decreased CO_2 provides insight into *H. pylori* natural competence regulation within the gastric mucosa. Our results indicate that *H. pylori* competence is highly sensitive to subtle changes that could be experienced within microenvironments of the mucous layer. These conditions of increased pH and reduced CO_2 can also be exploited to optimize transformation efficiency for generating isogenic mutants in the laboratory.

ACKNOWLEDGMENTS

The *H. pylori* SS1 sequence, used for the construction of pOMPSS1 and pIGSS1, was generously provided by Jenny Draper and Karen Ottemann. We also thank Lori Hansen for designing several primers.

This work was supported by grants R01 AI081037 and R01 AI070803 from the National Institutes of Health to Jay V. Solnick.

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