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NATURAL TRANSFORMATION INCREASES THE RATE OF ADAPTATION IN THE HUMAN PATHOGEN *HELICOBACTER PYLORI*

David A. Baltrus,[1,2] Karen Guillemin,[3,4,6] and Patrick C. Phillips[1,5,6]

[1]*Center for Ecology and Evolution, University of Oregon, Eugene, Oregon 97403*

[3]*Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403*

[4]*E-mail: guillemin@molbio.uoregon.edu*

[5]*E-mail: pphil@uoregon.edu*

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Gene exchange between individuals can lead to profound evolutionary effects at both the genomic and population levels. These effects have sparked widespread interest in examining the specific adaptive benefits of recombination. Although this work has primarily focused on the benefits of sex in eukaryotes, it is assumed that similar benefits of genetic exchange apply across eukaryotes and prokaryotes. Here we report a direct test of this assumption using the naturally transformable human gastric pathogen *Helicobacter pylori* as a model organism. We show that genetic exchange accelerates adaptation to a novel laboratory environment within bacterial populations and that a general adaptive advantage exists for naturally transformable strains when transfer occurs among conspecific backgrounds. This finding demonstrates that there are generalized benefits to adaptation in both eukaryotes and prokaryotes even though the underlying processes are mechanistically different.

KEY WORDS: **Adaptation, experimental evolution, genetic exchange, *Helicobacter pylori*, natural transformation.**

Cross genome comparisons have demonstrated an extensive role for horizontal gene transfer in prokaryotic evolution. The benefits of gene exchange between species are often evident because novel phenotypes with strong selective effects that might be practically inaccessible through mutation over short time scales (i.e., antibiotic resistance genes and whole catabolic pathways) can be introduced into genomes in a single step (Dobrindt et al. 2004). Gene transfer may also act as a cohesive force within species by allowing allelic variation to be shared across divided populations (Fraser et al. 2007). In these cases, the evolutionary advantage of horizontal transfer would arise specifically because both mutation and gene exchange introduce genetic variation at the population level. However these same genetic exchange systems may also provide significant evolutionary advantages within populations through recombination between existing alleles. Although many studies have documented high levels of recombination within bacterial species, even between ubiquitously present housekeeping genes (Narra and Ochman 2006), the adaptive benefits of exchange are less clear within populations than between. Although much of the focus on genetic exchange within prokaryotes has been on the movement of genes across broad taxonomic domains, the evolutionary forces shaping the maintenance and evolution of the exchange systems themselves must be operating within populations.

The idea that prokaryotic recombination can significantly alter evolutionary dynamics is often assumed to hold true (i.e., Kang and Blaser 2006), as opposed to being an epiphenomenon of other direct functions for such systems (Redfield 2001), because extensive theoretical and experimental investigations have identified adaptive benefits for the analogous process of eukaryotic sex (Colegrave 2002; Otto and Lenormand 2002; Rice 2002; Goddard

[2]Present address: Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599.
[6]To whom correspondence should be addressed.

et al. 2005). These previous eukaryotic studies have shown that the evolutionary advantage for sex lies in the breakdown of linkage disequilibrium (Otto and Lenormand 2002; Otto and Gerstein 2006), which is generated within populations by the loss of fittest genotypes by genetic drift (Muller's ratchet), by mutation limitation within the population (clonal interference), by population subdivision, or by inherent epistatic interactions between mutations. General disadvantages for sex applicable to prokaryotes include the breakup of beneficial combinations of alleles as well as direct fitness costs (i.e., energetic costs or sexually transmitted disease). One should note that many of these hypothesized benefits are dependent on parameters that vary greatly within natural populations so that the potential advantages of sex are very small or nonexistent under many plausible conditions (Otto and Gerstein 2006; Kouyos et al. 2007). Furthermore, many of the hypothesized general benefits for recombination explicitly rely upon specific ranges for population level parameters (such as N_e) that can differ dramatically between prokaryotes and eukaryotes (Lynch and Conery 2003; Otto and Gerstein 2006).

Of the several mechanisms that allow for bacterial gene exchange, natural transformation through DNA uptake may have extensive and generalized effects within populations. Selection for the presence of natural transformation systems is suggested by the phylogenetic diversity of competent bacteria as well as the observation that structural components of transformation systems have independently evolved at least twice (Lorenz and Wackernagel 1994; Smeets and Kusters 2002). Unlike conjugation and transduction, which did not evolve for recombination but are by-products of the action of parasitic elements, genes controlling the uptake of DNA directly from the extracellular environment are encoded entirely by the bacterial chromosome (Lorenz and Wackernagel 1994; Redfield 2001) so that the evolutionary maintenance of recombination is not enhanced by selection on secondary elements like plasmids or phage. Furthermore, the benefits of horizontal transfer across species are limited because incorporation of DNA by natural transformation requires a minimum level of homology to sequences already present within the recipient genome (Lorenz and Wackernagel 1994). Novel genes must therefore be flanked by sequences conserved in the recipient to be exchanged. On the other hand, because recombination requires only minimal levels of homology, the effects of transformation could be much more highly localized than conjugation or transduction.

Although sex differs dramatically in mechanism from the three major forms of prokaryotic gene exchange (transduction, conjugation, and natural transformation), in a population genetics sense all of these forms involve the redistribution of alleles across genetic backgrounds. One can therefore use studies concerning sex in eukaryotes as a starting point for understanding bacterial gene exchange but should be cautious of how mechanistic differences affect evolutionary interpretations. The most relevant evolutionary difference between eukaryotic sex and natural transformation is that transformation of cells takes place through the incorporation of genetic material from an extracellular free DNA pool. DNA enters this pool either through active processes or by cell lysis (Lorenz and Wackernagel 1994), and there is great potential for the process of DNA release to bias the adaptive effects of natural transformation. For instance, because the free DNA pool can be generated by cell death, DNA from this pool possibly contains a higher number of deleterious mutations on average than living cells so that transformation could lower population fitness (Redfield 1988). Conversely, beneficial mutations that prolong cell survival could be underrepresented or even absent from the DNA pool and thus competent populations might at best only have equal rates of adaptation as noncompetent strains. Mutations or selfish elements increasing the rate of cell lysis could also preferentially fix in populations undergoing transformation despite detrimental effects on fitness (Draghi and Turner 2006). Lastly, the DNA pool potentially includes alleles of loci from unrelated or noncompetent strains that modify the level of competence. Transformation by these fragments could lower or eliminate the overall level of competence and allow noncompetent genotypes to inherently rise in frequency within a mixed population if not balanced by direct selection for functioning transformation systems (Redfield et al. 1997). The evolutionary advantages of natural transformation should generally overlap with those of sex as long as genotype frequencies within the DNA pool are equal to those within the living population. However, there are currently no estimates of genotypic frequencies within the free DNA pool even though, as with the cases described above, deviations in these frequencies from those of the living population (linkage disequilibrium between living and dead cells) lead to dramatic differences in the evolutionary outcomes of gene exchange. Along these lines, it is intriguing that a recent experiment attempting to measure the adaptive advantage of competence within populations of *Acinetobacter* failed to show any evolutionary benefit even with conditions promoting linkage disequilibrium and in fact found that competence decreased during adaptation (Bacher et al. 2006). Thus, even though previous experiments have verified the evolutionary benefits of sex in eukaryotes, further tests are necessary to understand the evolutionary dynamics of natural transformation within prokaryotic populations.

We have established a laboratory system using the human gastric pathogen *Helicobacter pylori* to test for the adaptive effects of genetic exchange within bacterial populations. *Helicobacter pylori,* the causative agent of gastric ulceration and an important risk factor for stomach cancer, is a gram-negative bacterium that inhabits 50% of the world's human population (Blaser and Atherton 2004). *Helicobacter pylori* is well suited for these studies because cells are naturally transformable in the laboratory

throughout different growth phases, with no known uptake sequences to bias incorporation of DNA (Saunders et al. 1999; Baltrus and Guillemin 2006). Although little is known about the mechanism of DNA transfer or exact physiology of competence induction in *H. pylori*, there is significant variation between strains in both the timing and number of peaks in competence during growth in the laboratory (Baltrus and Guillemin 2006). Most importantly, recombination, likely by natural transformation, is demonstrated to have a major effect on the structure of natural populations of *H. pylori* (Suerbaum et al. 1998; Gressmann et al. 2005) and has been demonstrated to occur within a single host during infection (Suerbaum and Josenhans 2007). Here we report the creation of the experimental laboratory system and provide the first direct evidence for an evolutionary advantage of natural transformation within a bacterial population.

Methods

BACTERIAL STRAINS AND MEDIA

A single colony isolate of *H. pylori* strain G27 (Xiang et al. 1995) was used to found all replicate transformable populations (Table 1 gives a list of all strains used). To our knowledge this isolate of strain G27 (G27DB1) has not been extensively passaged in the laboratory and certainly has not experienced passage under constant conditions as described below. The nontransformable ancestor was created by the insertion of a *kansacB* cassette into the *comH* locus of strain G27DB1 between base pairs 660 and 977 (G27DB1KC) and was used to found five replicate lines. The *comH::kansacB* construct was also transformed into a single colony isolate of experimental transformable line S2 at generation 960 (S2175I1) to quantify fitness interactions between the evolved backgrounds and the *comH* allele. The control strain for all fitness assays (G271175SRKC) was a streptomycin-resistant derivative of a generation 960 isolate from line S4 that had been transformed with the *comH*::*kansacB* knockout allele. This strain was chosen as the common control because it grows more reliably than the ancestor under laboratory conditions. Recipes for liquid media and blood agar (BA) plates are as in Baltrus and Guillemin (2006). Selective plates were made with 10 μg mL^{-1} streptomycin, 25 μg mL^{-1} kanamycin, or 15 μg mL^{-1} rifampicin.

LABORATORY PASSAGE CONDITIONS

Laboratory evolution cultures were grown at 37°C in 5mL liquid cultures inside of 50-mL Falcon tubes. The Falcon tubes were then placed in racks inside two sealed containers with microaerobic sachets (Oxoid) used to create suitable environmental conditions. Each ancestral background was represented within both containers and lines were consistently maintained inside the same container with their positions relative to the sachet randomized each day. After 24-h growth, strains were serially passaged with a 1:50 dilution. A 500 μl sample from each population was frozen every five days in 1 mL of *H. pylori* freezing media. After every 25 days of continuous passage, strains were restarted from their frozen samples by passage for three days on BA plates, resuspension in liquid media, and 1:100 dilution to restart the serial passage cycle. Approximately 50 μl of the frozen stock was used to restart each of the lines, so that the populations underwent a brief bottleneck to $\sim 1 \times 10^6$ cells during the freeze/thaw cycle. This bottleneck was included in our estimates of effective population size for the populations. Without this freezing step we found that lines would completely fail to grow on occasion when transferred to freshly made batches of growth media. Although this step greatly aided in continual maintenance of the lines, it was also included because freezing is always ultimately part of the competitive fitness assay. A schematic of the laboratory passage scheme is provided in online Supplementary Figure S1.

Table 1. Strains used in this study.

Strain	Description	Reference
G27DB1	Single colony isolate of strain G27 (competent ancestor)	This study
G27DB1KC	G27DB1 with *comH* disrupted by *KansacB* (noncompetent ancestor)	This study
G27175SRKC	Streptomycin-resistant isolate of G27DB1 descendant S4 after 960 generations laboratory passage with *comH* disrupted by *KansacB* (control strain for fitness assays)	This study
G27*traG::KansacB*	Disruption of *traG*/HP1006 with *KansacB* cassette	(Baltrus and Guillemin 2006)
G27*cagA::cat*	Chloramphenicol-resistant isolate (*cagA* disrupted by *cat*)	(Salama et al. 2001)
G27*traG::KansacB*∇*comH*	G27*traG::KansacB* with deletion of *comH*	This study
G27*cagA::cat*∇*comH*	G27*cagA::cat* with deletion of *comH*	This study
G27S2175I1	Single colony isolate of G27DB1 descendant line S2 after 960 generations of laboratory adaptation	This study
G27S2175I1*comH::KansacB*	Single colony isolate of laboratory adaptation line S2 after 175 days passage with *comH* disrupted by *KansacB*	This study

COMPETITIVE FITNESS ASSAYS

Prior to the fitness assay, strains were started from frozen samples as during laboratory passage, acclimated to laboratory growth during three passage cycles, and then mixed in a 1:1 ratio according to OD_{600}. A dilution was plated in triplicate on nonselective BA plates to measure total population size. After 24 h of growth of this mixed culture, an additional sample was plated in triplicate onto nonselective BA plates. After five days of incubation of the hour 0 and hour 24 nonselective plates, colonies were replica plated to BA plates containing streptomycin and kanamycin. We define fitness as selection rate per day between the control strain and the experimental strain (Travisano and Lenski 1996). Selection rate compared to the control strain was calculated for each experimental strain and ancestor in this assay. This measurement represents the difference between the natural logarithm of population growth rates for each experimental line and its ancestor, and thus the fitness of each ancestral strain is normalized to a value of zero. Because the ancestral lines differ slightly in selection rate compared to the control strain, fitness for each evolved strain was calculated by taking the difference between selection rate of each evolved line to the control line and adding the difference between the specific ancestor and the control.

Fitnesses of the ancestral lines were measured in six independent blocks for a total of 40 replicates per line. Fitnesses of experimental lines at each evolved time point were measured as whole population samples with three independent blocks and four replicates of each line per block. Fitness assays between a single colony isolate of generation 960 experimental line S2 (S2175I1) and its derived *comH* knockout (S2175ICH) were carried out as above with three independent blocks of six replicates each. A schematic of the competitive fitness assay is provided in online Supplementary Figure S2. Direct competitions between wild-type and *comH*- ancestral lines proved difficult to interpret because direct assays consistently overestimated growth of the phenotypically marked line regardless of the locus disrupted (data not shown). This bias is likely the product of higher numbers of colonies on plates from the 24-h time point as well as replica plating. Bias in the fitness assay should not be a problem for fitness comparisons in this article because growth of all experimental lines is measured in the same way against a common control.

MEASUREMENT OF RATES OF MUTATION AND TRANSFORMATION

Mutation rates to resistance for each antibiotic (rifampicin or streptomycin) were calculated using three, four (ancestral transformable strain on streptomycin), or six (ancestral transformable strain on rifampicin) replicated fluctuation tests. Cells were acclimated to laboratory conditions as in Baltrus and Guillemin (2006), after which 20 (rifampicin) or 30 (streptomycin) independent 1-mL cultures were inoculated with approximately 1000 cells from the starter culture and placed inside of a sealed container on a rotary shaker. After 38–44 h, dilutions from three (rifampicin) or four (streptomycin) cultures were plated to measure population sizes, with the remaining cultures each plated in totality as 500 μL aliquots on selective agar. Mutation rates were calculated using fluctuation test analysis software provided by Paul Sniegowski (University of Pennsylvania). Rates were generally 1 log higher when the median method was used to calculate mutation rates to rifampicin resistance (Bjorkholm et al. 2001), however, there were no treatment-specific effects to this difference. To measure transformation rates between strains, two strains phenotypically marked by different antibiotic resistance alleles (G27*traG::kansacB* and G27*cagA::cat*) were acclimated to laboratory passage as above and then mixed in a 1:1 ratio (according to OD_{600}) to a total volume of 100 μL. There were three replicate cultures for each trial. The mixed cultures were allowed to grow for 24 h under the defined laboratory passage conditions after which time samples were plated on BA plates to determine total population size and the number of recombinants, and a sample was frozen at −80°C. Total population size was estimated by dilution plating on nonselective BA plates, and the number of transformants was measured by plating 200 μL of the mixed cultures on chloramphenicol + kanamycin BA plates. The mixed cultures were then restarted from the frozen samples as above and passaged for an additional 24 h after which time additional sampling for total population size and number of transformants occurred. As a negative control, experiments were performed similarly in genetic backgrounds containing a deletion of *comH*. Assays of transformation rate were performed twice independently.

STATISTICAL METHODS

A *t*-test assuming unequal variance and different sample sizes was used to test for the difference in mutation rates between wild-type and *comH*- lines. An analysis of variance (ANOVA) model was used to compare fitnesses of the ancestral strains, with genetic background as a fixed effect and block as a random effect. Differences in fitness gains between competent and noncompetent lines were compared at each generation using a nested ANOVA model with line as a random effect nested within ancestor as a fixed effect and block as a random effect. A combined ANOVA model was used to compare the fitnesses of evolved lines at generations 360, 690, and 960, with ancestor as a fixed effect, line as a random effect nested with ancestor, generation as a fixed effect, and block as a random effect nested within generation. LS mean contrasts were used within this combined ANOVA model to compare transformable and nontransformable strains at both 690 and 960 generations. An ANOVA model was used to compare S2175I1 and its *comH* derivative, with background as a fixed effect and block as a random effect. Variances between lines within treatments (competent or noncompetent) were compared using a

Levene's test in which in the absolute deviation of each line mean to their treatment mean was compared using a *t*-test.

Results

PHENOTYPIC CHARACTERIZATION OF ANCESTOR STRAINS

Isogenic lines of *H. pylori* strain G27 were created that were either competent or noncompetent for natural transformation under laboratory conditions through disruption of the *comH* locus. Although the exact function of *comH* is not known (Smeets et al. 2000), we have not found a significant difference in fitness between wild-type and *comH* knockout lines after one day of growth (Table 2). However, a significant block effect was present in this assay ($F_{5,73} = 7.42$, $P < 0.001$) likely due to sensitivity of both the experimental and control strains to subtle environmental variability during growth in the laboratory. Because transformation can potentially increase mutation rate (Grist and Butler 1983), we further measured mutational rates in both of the ancestral lines with replicated fluctuation tests and selection on either rifampicin or streptomycin. We found no significant differences ($t_5 = -0.89$, $P = 0.79$ for rifampicin; $t_3 = -0.54$, $P = 0.689$ for streptomycin) between the ancestral strains in mutation rate to either antibiotic resistance (Table 2).

RATES OF NATURAL TRANSFORMATION DURING LABORATORY PASSAGE

Disruption of *comH* completely eliminates the ability to take up and be transformed by extracellular DNA (Smeets et al. 2000). Under our defined laboratory conditions, we found that no measurable genetic exchange takes place between strains in which *comH* has been deleted (Table 2). Furthermore, our design allowed for exchange of markers in the wild-type background. Natural transformation occurred at a highly variable rate under normal passage conditions, but this passage design also allowed for consistently high periods of natural transformation between cells every 140 generations during freezing and restarting the lines.

Table 2. Phenotypic comparison of ancestral lines.

Phenotypic measurement	Noncompetent ancestor	Competent ancestor
Selection rate to	−0.475[1]	−0.512
control strain	(0.042)	(0.042)
Mutation rate (per cell per division)		
$\mu_{Rifampicin}$	2.45×10^{-8}	2.98×10^{-8}
	(0.46×10^{-8})	(0.39×10^{-8})
$\mu_{Streptomycin}$	3.45×10^{-10}	4.34×10^{-10}
	(1.41×10^{-10})	(0.77×10^{-10})
Transformation rate (per cell per passage)		
During serial passage	0[2]	1.27×10^{-7}
		(2.09×10^{-7})
Starting from frozen	0[2]	6.06×10^{-6}
		(0.22×10^{-6})

[1] 1 SE is shown in parentheses beneath each number.
[2] Limit of detection = 3.0×10^{-9} per cell.

NATURAL TRANSFORMATION INCREASES THE RATE OF ADAPTATION

For each line we measured competitive fitness relative to a control strain after 360, 690, and 960 generations of laboratory passage and calculated the level of adaptation relative to the ancestral strains. We define fitness as selection rate per passage as in Travisano and Lenski (1996), which represents the natural log difference in population growth rates between each experimental line and its ancestor. There was no significant adaptive difference between the backgrounds after 360 generations (Fig. 1A), but we find that competence provides strains with an average fitness advantage of 0.14 (0.55 for competent and 0.41 for noncompetent) and 0.10 (0.72 for competent and 0.62 for noncompetent) after 690 and 960 generations of adaptation, respectively, under these laboratory conditions. These relative fitness advantages are equivalent to a 15% difference in population growth during one passage for the generation 690 lines and an 11% difference at generation 960. When the fitness gains of competent and noncompetent lines are compared separately at each generation, the 11% difference between these treatments at generation 960 ($F_{1,8} = 8.15$, $P = 0.021$) was significant. However, using this statistical design, the 15% advantage measured at generation 690 did not fall below the level of significance ($F_{1,8} = 3.68$, $P = 0.091$), likely due to high within-line variance. One should also note that significant block effects were found at both generation 360 ($F_{1,8} = 18.260$, $P < 0.0001$) and 690 ($F_{1,8} = 5.25$, $P < 0.0001$), but not for generation 960 ($F_{1,8} = 0.2344$, $P = 0.7915$).

To further test for an overall effect, we analyzed fitness gains together for all three generations and performed post hoc contrasts at the later time points where we expected to see the adaptive advantage of competence. The replicated competent strains showed larger increases in fitness than the noncompetent strains when the combined data for generations 690 and 960 were analyzed ($F_{1,16} = 9.09$, $P = 0.008$). Furthermore, analyzed as a contrast within the complete model using all samples to estimate the within-treatment variance, the difference at generation 690 was more clearly resolved ($F_{1,16} = 6.02$, $P = 0.025$).

Competitive fitness assays were initiated by mixing experimental strains and the control strain in a 1:1 ratio according to OD_{600}. It is possible that the relationship between colony-forming units (CFU) and OD_{600} changed during the course of laboratory passage, and such a change could affect fitness measurements if beneficial mutations display frequency dependence. We have found that the initial ratio of experimental to control strain CFU

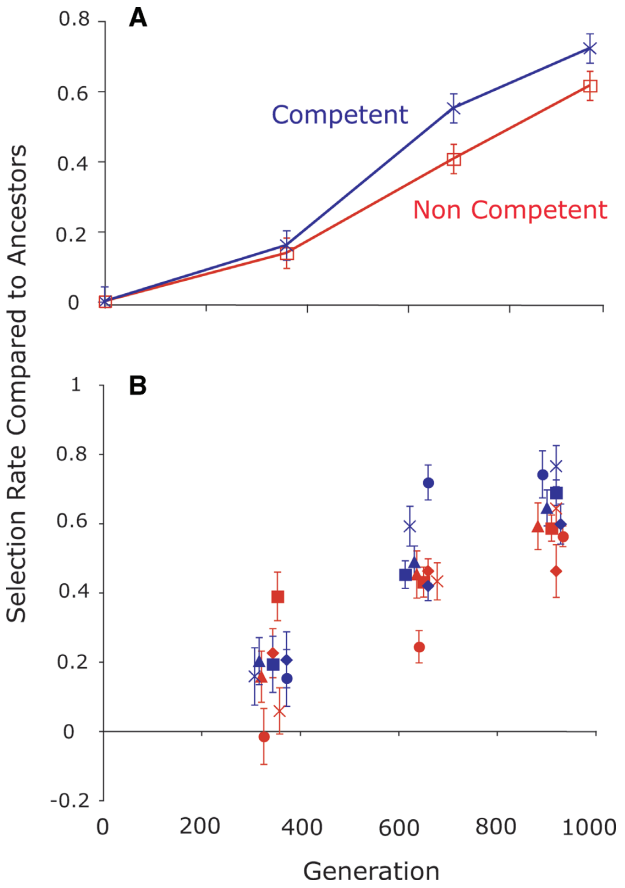

Figure 1. Adaptation to a novel environment is faster in strains able to take up exogenous DNA (competent for natural transformation) than in those that cannot (noncompetent). Fitness is defined as the selection rate, the difference between the natural logs of population growth rate, between a given line and its nonadapted ancestor. The ancestral value is set at a value of zero. Noncompetent lines are colored red (boxes), while competent lines are colored blue (x's). (A) The least squares mean selection rate for competent and noncompetent backgrounds are plotted for each time point. (B) The average selection rate of each experimental line is plotted for each time point. Points are slightly displaced on the x-axis for clarity in Figure 1B. In each graph error bars represent 1 SE calculated from the whole model. In Figure 1B, data for individual lines within each background are shown as x's for line 1, circles for line two, triangles for line 3, squares for line 4, and diamonds for line 5.

counts in all fitness assays is higher in lines that have experienced more extensive laboratory passage (0.346 for generation 360, 0.421 for generation 690, and 0.459 for generation 960; $F_{1,418} = 502.46, P < 0.0001$) indicating that the number of CFUs for a given OD_{600} value increased as a function of laboratory passage. Although calculation of the exact magnitude of fitness increases could be affected by the change in this relationship, we found no difference in this value between competent and noncompetent lines as a whole (0.409 for noncompetent, 0.412 for competent; $F_{1,3} = 0.178, P = 0.699$) such that the overall comparison in adaptive rates between these two backgrounds should not be affected.

COMPARISON OF VARIANCE BETWEEN GENETIC BACKGROUNDS

The mean fitness for each line at each generation is plotted in Figure 1B. We compared variance in fitness between lines within both competent and noncompetent backgrounds after 360, 690, and 960 generations of laboratory passage. Even with very little power to detect such a difference given this experimental design, variance in fitness was significantly higher ($t_4 = 2.36, P = 0.038$) between the noncompetent lines after 360 generations of passage ($s^2 = 0.03$) than between the competent lines ($s^2 = 0.00076$). However, there was no difference between lines within each genetic background at either generation 690 (0.01 and 0.018 for wild type and *comH*- respectively; $t_8 = -0.93, P = 0.81$) or 960 (0.0054 and 0.0058; $t_8 = -0.33, P = 0.62$).

FITNESS INCREASE IS NOT DEPENDENT ON *comH*

To determine whether higher rates of adaptation in the naturally transformable lines was directly caused by the presence of a wild-type *comH* allele, as opposed to the expected effect of transformation through redistribution of genetic variation during adaptation, we first isolated a single high fitness colony from experimental line S2 at generation 960. We then disrupted *comH* in this isolate using the same antibiotic resistance cassette used to create the noncompetent ancestor. Disruption of *comH* did not cause a significant change in fitness of this high fitness isolate ($F_{1,32} = 1.82, P = 0.672$) (Fig. 2). The fitness advantage seen in the transformable lines was therefore not dependent on the presence of a functioning *comH* locus.

Discussion

EXPERIMENTAL EVOLUTION IN *H. PYLORI*

We have developed a system to quantify the evolutionary advantage of recombination by natural transformation within populations using the human gastric pathogen *H. pylori*. We tested for differences in the rate of adaptation between competent and noncompetent strains during adaptation to a completely novel environment for *H. pylori*, laboratory passage. Our design allows for approximately 5.5 generations of growth per day (1:50 dilution during passage) with effective population sizes $>1 \times 10^7$ cells. Selection on *H. pylori* in the wild likely maximizes parameters such as transmission between hosts, survival during acidic stress within hosts, and avoidance of immune response (McGowan et al. 1996; Falk et al. 2000). In contrast, selection in the laboratory environment likely maximizes parameters such as cell division rates and survival at high cell density (Lenski et al. 1998; Cooper

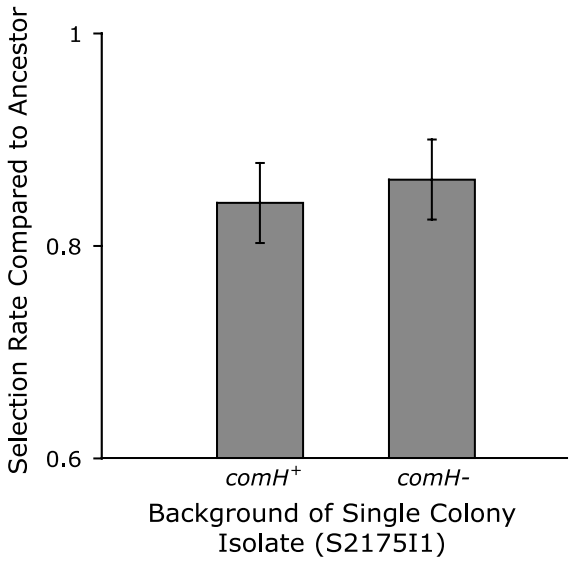

Figure 2. Fitness increase in the competent lines was not dependent on presence of the wild-type *comH* allele. A high fitness single colony isolate from one evolved competent strain at generation 960 was transformed with the *comH::kansacB* allele, and fitness of these knockout derivative was measured in relation to the untransformed line. Error bars show 1 SE.

et al. 2003). Our experiment thus directly tests for a generalized benefit of genetic exchange by transformation during adaptation to a novel environment rather than an evolved effect that might be specific to *H. pylori* cultures growing within the host. Furthermore, although the adaptive benefit of bacterial gene exchange is apparent when completely novel phenotypes of strong selective effect are transferred, our system tests for adaptive effects of natural transformation in populations that likely contain multiple circulating beneficial mutations of varying fitness effects and thus more closely approximates microevolution in a natural environment.

To create competent and noncompetent strains we isolated a single colony of strain G27 and disrupted the *comH* locus by the insertion of a cassette providing kanamycin resistance. Disruption of *comH* completely eliminates the ability to transform extracellular DNA (Smeets et al. 2000), and we have found that no genetic exchange occurs between *comH-* strains under laboratory passage conditions (Table 2). In contrast we found that recombination (1.27×10^{-7} per cell per passage) does take place in transformable cells under long-term passage conditions. The variability of this rate likely reflects the dependence of transformation on two stochastic variables, release and subsequent transformation by marker DNA. During periods where the lines were restarted from frozen (which occurred every 25 days), we found the potential rates of transformation to be uniformly high (6.06×10^{-6} per cell per passage). This increase in the rate of transformation could potentially be explained by increased competence while restarting the lines from frozen, higher cell densities during plate growth and thus more DNA released, or increased release of DNA due to lysis of cells during freezing.

In some naturally transformable bacteria, competence is induced under starvation conditions and DNA is used as a nutrient (Redfield 2001). It is possible that natural competence is used as a form of nutrition in *H. pylori*, however, we find no evidence that loss of competence negatively affects the fitness of ancestral *H. pylori* strains under laboratory growth (Table 2). Our experimental system thus allows for controlled serial passage of populations with and without genetic exchange, without large-scale phenotypic differences or any other experimental or physiological manipulation.

THE EVOLUTIONARY BENEFIT OF NATURAL TRANSFORMATION

We passaged five replicates of the competent and noncompetent ancestors for approximately 1000 divisions in batch culture. As one can see in Figure 1A, both backgrounds were able to adapt to the laboratory environment with fitness increases becoming apparent at the population level after 360 generations of growth. After 960 generations, the average fitnesses of the competent (0.72) and noncompetent (0.62) lines are equivalent to increases of population growth rate of 105% and 86%, respectively, during one passage when compared to their ancestors. This finding is not surprising, as clonal populations of *E. coli* have previously been shown to adapt to the laboratory environment quite readily (Lenski et al. 1991). We further find that the populations with genetic exchange had 15% and 11% higher rates of population growth than fully clonal populations after 690 and 960 generations of passage, respectively. Although it is difficult to compare across such diverse experiments, the average advantage of the competent lines appears similar in magnitude to that found for sexual reproduction in yeast (Goddard et al. 2005). These results directly demonstrate that natural transformation significantly increases the rate of adaptation to a novel environment within a bacterial population.

Recombination has been hypothesized to act as a homogenizing force between populations (Fraser et al. 2007). We found that variance in fitness between lines was significantly higher for the noncompetent lines at generation 360 than for the competent lines. Especially during the initial stages of adaptation, where effect size of beneficial mutations is expected to be relatively large, population level fitness is dependent on the order that mutations arise as well as relative selection coefficients between the backgrounds containing different beneficial mutations. Natural transformation could allow these competing beneficial mutations to be recombined into the same genetic background (Cooper 2007). Noncompetent backgrounds could be susceptible to getting temporarily stuck at local fitness peaks because some classes of beneficial mutations could occur more readily than others. Ultimately,

given enough time, genotypes stuck at local fitness optima could be surpassed by higher fitness allelic combinations. In support of this hypothesis, between line variance is much more similar for noncompetent and competent backgrounds at both later generations sampled.

ADAPTATION AND MUTATION RATES

Interpretation of these long-term experiments depends upon the assumption that ancestral lines behave similarly to each other in evolutionarily relevant variables other than the rate of transformation. Specifically, mutation rate is known to substantially affect the rate of adaptation and natural transformation of DNA was reported to be mutagenic in both *Bacillus subtilis* (Yoshikawa 1966) and *Streptococcus pneumoniae* (although in *S. pneumoniae* the effect was largely due to heterologous DNA) (Grist and Butler 1983). To account for this effect we measured mutation rates of both ancestral backgrounds. One should keep in mind that it is extremely difficult to accurately measure mutation rates, especially so for a bacterium that naturally grows only in a host, due to the sensitivity of fluctuation tests to slight differences in environmental effects and growth (P. Sniegowski, pers. comm.).

Although measured mutation rates for resistance to two antibiotics are slightly higher in the competent background than the noncompetent *comH-* derivative, the rates are not significantly different between strains and we have no reason to reject the null hypothesis. Although it is possible that the evolutionary advantage of transformation can be explained by this slightly higher mutation rate, we do not know the spectrum of selective effects for beneficial mutations during laboratory passage of *H. pylori* and therefore cannot accurately determine how such a difference affects adaptation. We can, however, provide estimates of the expected difference in the number of mutations during the course of this experiment. Because the genome size of strain G27 is approximately 1.6 Mb, we estimate that the mutation rates per genome per division for the two ancestral strains (wt and *comH-*) are 6.94×10^{-4} and 5.52×10^{-4}, respectively. These numbers are likely underestimates because they are based upon per nucleotide mutation rates to streptomycin and do not include insertions, deletions, and frameshifts. One should also note that our measurements of mutation rates are tenfold higher if calculated by the median method as used previously with other *H. pylori* strains (Bjorkholm et al. 2001). With an estimated N_e of 3×10^7, it will take approximately 77 and 97 divisions for the competent and noncompetent lines to experience a single mutation at every nucleotide position. Therefore, if the difference in mutation rate truly exists, at the end of 960 generations of passage the competent lines will have experienced approximately three more mutations on average at each nucleotide of the genome, the majority of which should be deleterious (Sniegowski et al. 2000).

CLONAL INTERFERENCE AND LABORATORY PASSAGE OF *H. PYLORI*

Segregating variation of alternative alleles within different genetic lineages is essential if natural transformation is to provide a significant evolutionary advantage through the redistribution of alleles. One of the most likely forces for generating such segregating variation within bacterial populations is clonal interference (Gerrish and Lenski 1998). Clonal interference occurs because multiple beneficial mutations arise within independent lineages within a population. As genotype frequencies of these independent lineages increase the relative fitness of the overall population increases, however, the complete fixation of either single lineage is slowed because these lineages ultimately have to outcompete each other. Recombination has been demonstrated to overcome the "speed limit" of clonal interference within evolving *E. coli* cultures (Cooper 2007). Moreover, the presence of clonal interference within the experimental *H. pylori* cultures would render the slight difference in mutation rates between transformable and nontransformable lines inconsequential because fixation of beneficial mutations would be a more limiting evolutionary step than generation of the alleles. For instance, under appropriate conditions, de Visser et al. were able to show that 33-fold increases in mutation rate had no effect within evolving populations of *E. coli* (de Visser et al. 1999).

Several lines of evidence suggest that our passage scheme provides sufficient conditions for multiple competing beneficial mutations to arise within these experimental *H. pylori* populations. Using our current estimates of N_e and the genomic mutation rates of the noncompetent lines, we find that approximately one out of every 600,000 mutations has to be beneficial in order for clonal interference to have a significant effect on adaptive rates under this laboratory passage scheme (Gerrish and Lenski 1998). Although this figure is slightly higher than the one in a million figure as calculated for the *E. coli* long-term evolution experiments, one should note that the genomic mutation rate we use excludes various classes of mutation so that this number is likely an underestimate. Second, the population genetic variables within our experimental *H. pylori* system compare fairly well with those for *E. coli* laboratory experiment ($\mu = \sim 10^{10}$ and $Ne = \sim 10^7$) that was used to demonstrate the presence and clonal interference as well as the negligible effects of higher mutation rates during laboratory passage (de Visser et al. 1999). Lastly, we have discovered isolates of *H. pylori* within these laboratory evolution lines that possess similar fitness levels but show phenotypic diversity in motility (data not shown). Although other explanations are possible for these isolates, such as frequency-dependent selection, their presence is suggestive of the potential for competing beneficial mutations to arise within these experimental *H. pylori* populations.

ALTERNATIVE CAUSES OF THE ADAPTIVE ADVANTAGE OF COMPETENCE

Clonal interference is by no means the only way in which genotypic linkage disequilibrium builds up within a population. It is possible that the measured advantage within the transformable lines is enhanced by the breakdown of disequilibrium generated by negative epistasis between beneficial mutations as well as through subtle population subdivision within these evolving cultures. Most other theories predicting evolutionary advantages for genetic exchange depend strongly on deleterious mutations reaching high frequencies in populations, which is unlikely in our cultures given the total length and conditions of passage (Kondrashov 1988). Effective population sizes under laboratory passage are on the order of 10^7 cells and the effect of genetic drift should therefore be weak.

A future goal of this research is therefore to identify the beneficial mutations within the system to firmly understand how these mutations and their associated backgrounds interact epistatically and at the population level. To this end we are currently sequencing the complete genome of *H. pylori* strain G27 (D. A. Baltrus and K. Guillemin, unpubl. data). The causes of this disequilibrium can then be sorted by following genotypic frequencies of the beneficial alleles within multiple populations over the span of laboratory passage as well as by measuring laboratory fitness of these beneficial mutations in the ancestral genomic background both alone and in combination.

GENE EXCHANGE AND ADAPTATION FROM INITIALLY CLONAL POPULATIONS

Linkage disequilibrium between genotypes is a prerequisite for recombination to provide an evolutionary advantage within a population. Previous studies using prokaryotes have addressed whether gene exchange is beneficial to a population when such exchange introduces genetic variation (Graham and Istock 1979; Souza et al. 1997). Coupled with extensive evidence from whole genome sequences, these studies have shown conclusively that gene transfer is a powerful evolutionary force when genotypes are under complete linkage disequilibrium (i.e., loci or alleles are present within one population but not another). Here we sought to address a slightly different question, specifically, could natural transformation function to accelerate evolution in a population where genetic variation was introduced solely by mutation. Therefore, that we found an advantage in the competent lines implies that other population level processes such as clonal interference or negative epistasis function strongly enough to generate an advantage for natural transformation. We were also interested in identifying if the various processes that contribute to gene exchange by natural transformation (generation of the DNA pool, physiological timing of competence and recombination) act to negate any potential advantage through the redistribution of mutations. It was conceivable, especially because a previous experiment failed to find an adaptive advantage for transformation (Bacher et al. 2006), that such biases in the mechanism of competence could cancel out evolutionary advantages even if linkage disequilibrium were present. We could not address both of these questions if we were to manipulate genotype frequencies within the population and DNA pool as had been done in previous experiments.

ALTERNATIVE EXPLANATIONS FOR THE ADVANTAGE OF COMPETENT LINES

Previous studies have speculated that the evolution of competence can be explained by direct selection to import DNA into the cell so that it can be used for nutritional purposes (Redfield 2001). If DNA were used as a nutrient or other directly selectable processes by *H. pylori* under these laboratory conditions, differences in the rates of adaptation could be explained by competent strains having access to a more complex environment. In effect, higher rates of adaptation in the transformable strains could be due to their ability to more efficiently exploit DNA as a nutrient. Furthermore, the exact biochemical function of the *comH* protein is currently unknown and there are currently no known orthologues found within other bacterial genomes (Smeets et al. 2000). We cannot explicitly rule out that *comH* functions in processes other than competence and that this function could affect the evolutionary trajectory. An alternative explanation for our experimental results would therefore be that the adaptive landscapes of wild-type and *comH*-backgrounds are not equivalent, and that the fitness difference between backgrounds therefore represents a comparison between two different adaptive peaks.

One test of these alternative hypotheses is to determine whether benefits of the accumulated mutations within the competent lines are dependent on the presence of a wild-type *comH* allele. To test for this epistatic relationship we transformed the *comH* knockout allele into a single colony isolate of one of the highest fitness transformable lines. If adaptation in this line depends on a functioning *comH* allele we would expect fitness measurements to be much lower in the transformed strains than their nontransformed counterparts. Instead we found that disrupting the *comH* locus in this generation 960 transformable line did not have a detrimental effect on fitness (Fig. 2). Disruption of *comH* in this line has approximately the same slight effect as in the ancestor. These results, along with the lack of phenotypic differences between competent and noncompetent ancestral strains under passage conditions, support the model that natural transformation provides a general evolutionary advantage.

Conclusion

There have been multiple tests of theories providing explanations for the adaptive advantage of eukaryotic sex. Application of

present theory on the evolution of sex to understand gene transfer within bacterial populations is dependent on the assumption that mechanistic differences between such systems do not bias evolutionary interpretation. Due to physiological and experimental complications bacterial researchers have only tested tangential predictions of the theories (such as transformation of homologous DNA allowing for a higher survivorship after UV irradiation) (Redfield 2001). Furthermore, mathematical modeling of evolutionary processes in bacteria is informative but practically impossible to apply at present to natural populations because of the difficulty of measuring the required parameters like the epistatic relationships between mutations, the effective recombination rate, and average number of mutations in the transformable DNA pool, as well as complexities introduced by population level mechanisms such as clonal interference.

Using a naturally transformable model organism, we demonstrate the presence of adaptive advantage for natural transformation within evolving prokaryotic populations. Because clonal interference should be present within this system, we believe that the measured adaptive advantage of the naturally transformable background is best explained by the ability of genetic exchange to bring together novel beneficial mutations and therefore to break up linkage disequilibrium (Fisher 1930; Muller 1932; Michod and Levin 1988). These findings suggest that generalized advantages for genetic exchange exist across prokaryotic and eukaryotic kingdoms despite extensive differences in the mechanics of exchange and population dynamics of the study organisms.

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