The Effect of Mismatch Repair and Heteroduplex Formation on Sexual Isolation in Bacillus

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ABSTRACT

In Bacillus transformation, sexual isolation is known to be an exponential function of the sequence divergence between donor and recipient. Here, we have investigated the mechanism under which sequence divergence results in sexual isolation. We tested the effect of mismatch repair by comparing a wild-type strain and an isogenic mismatch-repair mutant for the relationship between sexual isolation and sequence divergence. Mismatch repair was shown to contribute to sexual isolation but was responsible for only a small fraction of the sexual isolation observed. Another possible mechanism of sexual isolation is that more divergent recipient and donor DNA strands have greater difficulty forming a heteroduplex because a region of perfect identity between donor and recipient is required for initiation of the heteroduplex. A mathematical model showed that this heteroduplex-resistance mechanism yields an exponential relationship between sexual isolation and sequence divergence. Moreover, this model yields an estimate of the size of the region of perfect identity that is comparable to independent estimates for *Escherichia coli*. For these reasons, and because all other mechanisms of sexual isolation may be ruled out, we conclude that resistance to heteroduplex formation is predominantly responsible for the exponential relationship between sexual isolation and sequence divergence in Bacillus transformation.

WHILE genetic exchange in bacteria is relatively rare, it is at the same time very promiscuous, so that bacteria are able to exchange genes with distantly related species (Maynard Smith *et al.* 1993; Cohan 1994). The frequency of genetic exchange between divergent species has been shown to depend on the DNA sequence divergence between them (Shen and Huang 1986; Roberts and Cohan 1993; Zawadzki *et al.* 1995). In the case of genetic transformation in *Bacillus subtilis*, a Gram-positive bacterium, there exists an exponential relationship between the reluctance of a recipient cell to incorporate foreign donor DNA and the DNA sequence divergence between donor and recipient:

$$\rho = 10^{\pi\phi}, \qquad (1)$$

where ρ is the reluctance to exchange genes, or sexual isolation; π is the DNA sequence divergence; and ϕ is the sensitivity of sexual isolation to sequence divergence (Roberts and Cohan 1993; Zawaszki *et al.* 1995).

This exponential relationship was recently observed in two other systems of recombination: natural transformation in Streptococcus, another Gram-positive bacterium (P. Zawadzki, J. Majewski, C. G. Dowson and F. M. Cohan, unpublished data), and *Hfr*-mediated conjugation in *Escherichia coli*, a Gram-negative bacterium (M. Vulic, unpublished data). Also, the frequency of intrachromosomal recombination in the yeast *Saccharomyces cerevisiae* decreases exponentially with the sequence divergence between recombining segments (S. Jinks-Robertson, unpublished data). Because the exponential relationship between sequence divergence and the reluctance to recombine has now been seen in phylogenetically diverse organisms and in different modes of recombination, we believe that this exponential relationship may be widespread throughout the microbial world. Here, we investigate the mechanisms underlying this relationship.

A successful genetic exchange event requires completion of all the following steps: (1) uptake of donor DNA by the recipient cell, (2) escape of the donor DNA from the recipient's restriction system, (3) formation of a donor-recipient DNA heteroduplex intermediate, (4) escape of the heteroduplex from the mismatch repair system, and (5) successful functioning of the donor gene product, which requires functional compatibility of the donor gene product with the recipient cell's genetic background and physiology (Smith 1988; Dubnau 1993; Matic *et al.* 1996). In principle, increased sexual isolation between divergent strains could be because of increased resistance in any of these five steps.

Experiments carried out by Matic *et al.* (1995) investigated the mechanism of sexual isolation in conjugation between *E. coli* and Salmonella. They found that (1) the frequency of matings between the two species is

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not significantly different from the frequency of matings within species, (2) the frequency of integration of foreign DNA is not significantly affected by the recipient's type II restriction/modification system, and (3) mutants deficient in the MutS mismatch repair protein exhibit a drastically reduced level of sexual isolation. They concluded that mismatch repair is the predominant barrier to recombination.

Matic *et al.* (1996) suggested a mechanism by which mismatch repair contributes to sexual isolation between divergent species. If mismatch repair detects a DNA base pair mismatch, it excises a long patch of the foreign DNA strand (which may be the size of the entire recombining molecule; Claverys and Lacks 1986). The strand is subsequently resynthesized according to the original template. Hence, the mismatch repair system is able to prevent foreign DNA from being integrated into the chromosome.

Here, we investigate the effect of mismatch repair on transformation frequency in *B. subtilis*, and we propose a mechanism for the exponential relationship between sexual isolation and sequence divergence. We have compared the relationship between sexual isolation and sequence divergence in two isogenic recipient strains, one that is wild type (1A96) and one that is deficient for the genes *mutS* and *mutL* of the mismatch repair system (PB1856; Ginetti *et al.* 1996). The proteins MutS and MutL are thought to comprise the entire mismatch repair system of Bacillus, and these proteins are homologous to the mismatch repair system responsible for sexual isolation in Escherichia conjugation (Ginetti *et al.* 1996). Our approach, based on Roberts and Cohan (1993), was to transform each recipient strain toward rifampicin resistance (coded by rif^R alleles of *rpoB*), by using donor DNA from rif^R strains with varying levels of divergence from the recipient.

MATERIALS AND METHODS

Strains: *B. subtilis* strains 1A96 (a derivative of strain 168) and 2A2 (a derivative of strain W23) were obtained from the Bacillus Genetic Stock Center (BGSC, Columbus, OH). The type strains of *B. amyloliquefaciens* (ATCC 23350) and *B. licheniformis* (ATCC 14580) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The type strain of *B. atrophaeus* (NRRL NRS-213) was obtained from the Agricultural Research Service Culture Collection at the National Center for Agricultural Utilization Research (Peoria, IL). The *B. subtilis* mismatch repair deletion mutant (PB1856) was kindly provided by Alessandra Albertini. This strain was constructed from strain 1A96 by replacing the entire *mutSL* operon with a chloramphenicol resistance cassette; this mutant therefore lacks all mismatch repair activity known to occur in Bacillus (Ginetti *et al.* 1996). All naturally occurring strains of *B. subtilis* and *B. mojavensis* were isolated from soil as described by Cohan *et al.* (1991). All strains used in this study are listed in Table 1.

The isogenic strains 1A96 and PB1856, derived from strain 168, were used as recipients in transformation experiments. Strains to be used as donors were chosen to represent six sequence similarity groups of Bacillus, with varying levels of sequence divergence from the recipients: the 168 and W23 groups of *B. subtilis*, *B. mojavensis*, *B. atrophaeus*, *B. amyloliquefaciens*, and *B. licheniformis* (Table 2).

Isolation of rifampicin-resistant mutants: For each strain to be used as a donor in transformation, rifampicin-resistant

TABLE 1

List of strains

Strain	Origin	Genotype
B. subtilis		
168 group		
1A96	BGSC	*pheA1 trpC2*
PB1856	A. Albertini	*pheA1 trpC2 ΔmutSL cat*
RO-NN-1	F. M. Cohan from soil	wt
W23 group		
RO-E-2	F. M. Cohan from soil	wt
2A2	BGSC	wt
B. mojavensis		
RO-C-2	F. M. Cohan from soil	wt
RO-H-1	F. M. Cohan from soil	wt
RO-QQ-2	F. M. Cohan from soil	wt
B. atrophaeus		
NRRL NRS-213	ARSCC	wt
B. amyloliquefaciens		
ATCC 23350	ATCC	wt
B. licheniformis		
ATCC 14580	ATCC	wt

BGSC, Bacillus Genetic Center; wt, Wild type; ARSCC, Agricultural Research Service Culture Collection at the National Center for Agricultural Utilization Service; ATCC, American Type Culture Collection.

mutants were isolated as described by Roberts and Cohan (1993).

Purification of genomic DNA: Genomic DNA was extracted and purified according to Cohan *et al.* (1991).

Estimate of sequence divergence at *rpoB*: The reported values of sequence divergence between the recipient strains (derivatives of strain 168) and the donor strains are based on the restriction digest data analysis of Roberts and Cohan (1993, 1995)

Transformation: The recipient strains were induced to be competent and were transformed toward rifampicin resistance with 3 μg/ml of genomic DNA extracted from rif^R mutants of donor strains (following Cohan *et al.* 1991). Sexual isolation (ρ of Equation 1) was calculated as the ratio of the homogamic transformation frequency, *i.e.*, using rif^R DNA derived from a mutant of the recipient strain, to the heterogamic transformation frequency (using a divergent donor's rif^R DNA).

Following Cohan *et al.* (1991), frequencies of transformation were calculated as the fraction of colony-forming units that were resistant to rifampicin, after accounting for spontaneous mutation toward rifampicin resistance (occurring in the absence of DNA at a frequency of 1.3×10^{-7} for 1A96 and 1.5×10^{-6} for PB1856).

RESULTS AND DISCUSSION

The effect of mismatch repair on sexual isolation: For both the parental strain (1A96) and its *mutSL* deletion mutant (PB1856), transformation frequencies decreased with donor–recipient sequence divergence (Table 2). Moreover, in both strains, sexual isolation (ρ) increased exponentially with sequence divergence (π) (Figure 1). The sensitivity coefficients (ϕ) were estimated from the slopes of the $\log_{10}$-transformed regressions as $\phi_{wt} = 21.37 \pm 1.25$ and $\phi_{mutSL-} = 17.92 \pm 0.58$ for recipients 1A96 and PB1856, respectively (Figure 1). An analysis of covariance showed these coefficients to be significantly different ($F_{1,11} = 10.04$, $P = 0.0089$). While the difference in sexual isolation between the mutant and wild type was most pronounced with *B. licheniformis* as donor, the mutant showed lower sexual isolation than the wild type across all donors: A sign test showed significant consistency across donors in the direction of the difference in sexual isolation (two-tailed sign test: $P = 0.016$, $N = 6$ sequence clusters used as donors). Therefore, the mismatch repair mutant is less sensitive to sequence divergence than is the wild type, as has been found for conjugation between Escherichia and Salmonella (Rayssiguier *et al.* 1989; Matic *et al.* 1995).

Nevertheless, in contrast to the case for Escherichia, most of the sexual isolation observed in Bacillus is not caused by mismatch repair. All donors, except for *B. licheniformis*, exhibit nearly identical values of sexual isolation with or without the presence of mismatch repair.

Let us consider the relative importance of mismatch repair and other mechanisms of sexual isolation. In the introduction, we suggested five possible mechanisms of sexual isolation. A successful recombination event requires escaping each of the relevant mechanisms. Thus, the probability of recombination (P_r) is equal to the probability of escaping mismatch repair (P_m) and escaping all other possible mechanisms (P_o). Assuming

TABLE 2

Transformation frequencies and levels of sexual isolation

Donor strain	Sequence divergence between donor and recipient at *rpoB*	$\log_{10}$(Transformation frequency)		$\log_{10}$(Sexual isolation)	
		Recipient 1A96	Recipient PB1856	Between donor and 1A96	Between donor and PB1856
B. subtilis					
168 group					
1A96	0	−2.045 ± 0.155[a]	−2.343 ± 0.209[a]	0	0
RO-NN-1	0.0124	−2.210 ± 0.258	−2.488 ± 0.178	0.208 ± 0.112[a]	0.132 ± 0.094[a]
W23 group					
2A2	0.0306	−2.495 ± 0.145	−2.682 ± 0.187	0.451 ± 0.042	0.326 ± 0.095
RO-E-2	0.0306	−2.346 ± 0.237	−2.636 ± 0.208	0.302 ± 0.083	0.356 ± 0.129
B. mojavensis					
RO-C-2	0.0457	−2.850 ± 0.206	−3.111 ± 0.199	0.805 ± 0.063	0.756 ± 0.166
RO-H-1	0.0457	−2.843 ± 0.239	−3.122 ± 0.183	0.799 ± 0.108	0.765 ± 0.185
RO-QQ-2	0.0486	−2.930 ± 0.183	−3.169 ± 0.097	0.886 ± 0.062	0.813 ± 0.083
B. atrophaeus					
NRRL NRS-213	0.0703	−3.286 ± 0.268	−3.593 ± 0.193	1.242 ± 0.122	1.236 ± 0.216
B. amyloliquefaciens					
ATCC 23350	0.0834	−3.645 ± 0.244	−3.900 ± 0.162	1.601 ± 0.114	1.545 ± 0.192
B. licheniformis					
ATCC 14580	0.1415	−5.420 ± 0.265	−4.952 ± 0.188	3.376 ± 0.127	2.597 ± 0.147

[a]Mean and standard error values for five experimental trials.

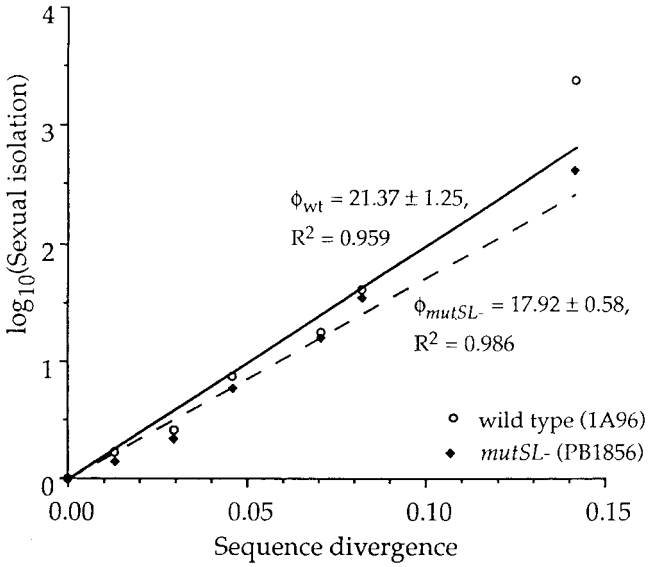

Figure 1.—The relationship between sexual isolation and sequence divergence for recipient strains wild type (1A96) and deficient (PB1856) for the MutS and MutL mismatch repair proteins. Each symbol represents the mean sexual isolation and sequence divergence levels over all donors from a particular sequence-similarity group, for each recipient. The solid line and the dashed line represent the best fit log–linear regression lines through the origin for the wild-type and *mutSL* recipients, respectively. The sensitivity parameters ϕ were estimated from the log–linear regressions.

that escaping each mechanism is an independent event,

$$P_r = P_m \cdot P_o.$$

Dividing P_r for heterogamic transformation by P_r for homogamic transformation (and substituting $P_m P_o$ for P_r) yields the following relationship for sexual isolation:

$$\rho = \rho_m \cdot \rho_o,$$

where ρ_m and ρ_o are the component contributions of mismatch repair and other mechanisms to sexual isolation.

We can now calculate the relative contributions of mismatch repair and other mechanisms to sexual isolation. For *B. licheniformis* as donor, $\rho = 2377$ (based on transformation with wild type), $\rho_o = 398$ (based on transformation with the mismatch repair mutant) and $\rho_m = \rho/\rho_o = 6.0$. Thus, even in the case of *B. licheniformis*, mismatch repair accounts for a sexual isolation factor of only 6, while other mechanisms account for the remaining factor of 400. This suggests an important role for a mechanism of sexual isolation other than mismatch repair.

Other mechanisms of sexual isolation: We next consider the four remaining mechanisms to explain why sexual isolation increases with sequence divergence.

First, successful genetic exchange requires uptake of donor DNA by the recipient cell, and so, sexual isolation may result because more divergent DNA is taken up less efficiently. However, it is known that the mechanism of DNA uptake is not sequence specific in either Bacillus (Dubnau 1991) or Streptococcus (Mejean and Claverys 1993). Therefore, the rate of uptake should be independent of sequence divergence. Similarly, the frequency of conjugation between *E. coli* and Salmonella is not significantly different from that of *E. coli*–*E. coli* matings (Matic *et al.* 1995). Thus, DNA uptake is not a source of sexual isolation in any of the bacterial systems known to have an exponential relationship between sexual isolation and sequence divergence.

Second, the recipient cell's restriction/modification system is a potential barrier to recombination. However, earlier studies carried out in our laboratory (Cohan *et al.* 1991; Zawadzki *et al.* 1995) have shown that restriction plays only a minor part in sexual isolation in Bacillus transformation (at most reducing heterogamic transformation by a factor of $\sim$6). Furthermore, the small effect of restriction in transformation introduces a constant amount of sexual isolation, regardless of the degree of sequence divergence. Restriction, therefore, cannot be responsible for producing the exponential relationship in Bacillus. Similarly, studies on *E. coli* and Salmonella conjugation have shown that sexual isolation is not significantly affected by type II restriction in the recipient (Matic *et al.* 1995).

Sexual isolation may also be caused by functional incompatibility of a donor gene's product with the physiology and biochemistry of the recipient cell. Functional incompatibility is most likely to prevent successful recombination at poorly conserved gene loci or between very divergent species. However, all of the donor–recipient combinations used in the model systems of sexual isolation are so closely related that protein functionality is likely to be conserved. In particular, in the *rpoB* gene used for transformation in Bacillus, all nucleotide substitutions between the recipient (*B. subtilis*) and donors appear to be synonymous (based on restriction digest analysis, Roberts and Cohan 1995).

Fourth, species may be sexually isolated because divergent donor and recipient DNA sequences have difficulty forming a heteroduplex. This is because *recA*-dependent recombination requires a small region of perfect identity shared by the recombining sequence and the recipient genome (Shen and Huang 1986); successful transformation would therefore be less likely between more divergent strains. We explore this one remaining explanation for the exponential relationship between sexual isolation and sequence divergence by proposing a mathematical model.

Model for the exponential relationship between sexual isolation and sequence divergence in Bacillus: In *E. coli*, *recA*-dependent recombination requires at least 40–50 consecutive base pairs of perfect identity between the recipient and donor at the 3′ end of the single-stranded

tail produced by the *recBCD* enzyme (Smith 1988). In Bacillus transformation, successful recombination also requires perfect identity between the recipient and the 3′ end of an invading donor strand (Smith 1988). However, in transformation, the donor DNA is fragmented and partially degraded upon entry into the cell (Smith 1988) so that the 3′ end of the invading strand is a random site from the donor genome. The probability of successful heteroduplex formation (P_h) is then the probability that a random donor strand has a 3′ end with n consecutive bases identical to the recipient. The probability (P_h) is a function of the sequence divergence (π) between donor and recipient:

$$P_h(\pi) = (1-\pi)^n. \quad (2)$$

We next consider the component of sexual isolation caused by resistance to heteroduplex formation (ρ_h). The value ρ_h is given by the probability of heteroduplex formation in homogamic recombination, *i.e.*, with zero donor–recipient divergence, divided by the probability in heterogamic recombination, in the absence of mismatch repair:

$$\rho_h = P_h(0)/P_h(\pi). \quad (3)$$

Substituting the values of P_h from Equation 2 into Equation 3,

$$\rho_h(\pi) = (1-\pi)^{-n}.$$

Taking logarithms of both sides,

$$\ln(\rho_h) = -n\ln(1-\pi).$$

We use the expansion

$$\ln(1-\pi) \approx -\pi + \pi^2/2 + \ldots$$

and make an approximation for small π by keeping only the first term of the expansion (this approximation is >92% accurate over the tested range of sequence divergence, *i.e.*, 1–14.15%), yielding

$$\ln(\rho_h) = n\pi$$
$$\rho_h = e^{n\pi}$$

Or, in base 10:

$$\rho_h = 10^{(n\pi/\ln 10)}. \quad (4)$$

The model thus predicts the observed exponential relationship between sequence divergence and sexual isolation.

By comparing Equations 1 and 4, we can now determine the sensitivity parameter ϕ of Equation 1 as a function of n:

$$\phi_h = \frac{n}{\ln 10}. \quad (5)$$

Here, ϕ_h is defined as the sensitivity of sexual isolation to sequence divergence when all sexual isolation results from difficulty in heteroduplex formation. Assuming that sexual isolation in our mismatch repair mutant was caused entirely by the difficulty in heteroduplex formation, our transformation experiments with *mutSL* yield an estimate of ϕ_h: $\phi_h = \phi_{mutSL-} = 17.85$. This assumption leads to the conclusion that the length of perfect identity required for *recA*-dependent recombination in *B. subtilis* is ~41 (using Equation 5). This is comparable to previous estimates between 40 and 50 bp, as observed for homologous recombination in *E. coli* (Smith 1988).

In summary, we began with five possible explanations for the exponential relationship between sexual isolation and sequence divergence, and all but the heteroduplex hypothesis has been ruled out. Moreover, our experimental results are consistent with the specific predictions of the heteroduplex hypothesis. The heteroduplex hypothesis predicts an exponential relationship between sexual isolation and sequence divergence, and the heteroduplex hypothesis yields an estimate of n (the size of the region of perfect donor–recipient identity required for recombination) that is consistent with independent estimates of this parameter. We therefore conclude that sexual isolation in Bacillus transformation is caused largely by the difficulty of heteroduplex formation with divergent recipient and donor DNA strands.

Comparison of Bacillus and other recombination systems: Although the exponential relationship between sexual isolation and sequence divergence has been observed in several systems, it is clear that the underlying molecular mechanisms are not in all cases the same. Our results indicate that heteroduplex inhibition is the most important cause of sexual isolation in Bacillus, while Matic *et al.* (1995) have demonstrated that mismatch repair is the primary barrier to interspecific recombination in *E. coli*. It will be interesting to determine why mismatch repair results in an exponential relationship between sexual isolation and sequence divergence in this other system. One possibility is that successful recombination requires every mismatched nucleotide site in a region to escape mismatch repair and that the probability of each site escaping detection is independent. In this case, the probability of successful recombination would be an exponential function of sequence divergence.

There may be several reasons why mismatch repair contributes so much less to sexual isolation in Bacillus than in Escherichia. First, studies on Streptococcus have shown that the homologous Hex system is effective at repairing single base mismatches produced by transformation, but it quickly becomes saturated (ineffective) if the recipient is transformed with more divergent (<1%) DNA (Humbert *et al.* 1995). It is possible that a similar saturation of the mismatch repair system occurs in Bacillus.

It has also been shown that in *E. coli*, the MutS protein is unstable and becomes degraded under starvation conditions (Feng *et al.* 1996). If the same is true in

Bacillus, this would imply that mismatch repair cannot be effective during transformation because *B. subtilis* becomes naturally competent for transformation only during starvation (Dubnau 1991).

Finally, resistance to heteroduplex formation may be higher in Bacillus transformation than in *E. coli* conjugation because much smaller segments are recombined in transformation than in conjugation. Because transformation involves the recombination of short DNA sequences (<4–8 kb; Zawadzki and Cohan 1995), the region of identity needs to be present within or very close to the locus of interest. On the other hand, with the much larger segments recombined in conjugation, the initiation of strand exchange may take place at highly conserved regions of the chromosome (such as *rrn* operons), far from the selected locus (Matic *et al.* 1995). Thus, donor–recipient sequence divergence is less likely to prevent heteroduplex formation in conjugation than in transformation.

If the relative importance of heteroduplex resistance is enhanced in modes of recombination involving smaller segments of DNA, then we can make the following predictions. First, because transformation in *B. subtilis* involves smaller segments than does transduction (Dubnau 1993), heteroduplex resistance should have a more important role in transformation than in transduction. Second, because transduction in *E. coli* involves much smaller segments than does conjugation (Margolin 1987; Willetts and Skurray 1987), heteroduplex resistance should have a more important role in *E. coli* sexual isolation in transduction than in conjugation. Furthermore, we predict that sexual isolation in *E. coli* will be greater for transduction than for conjugation because, in transduction, both mismatch repair and heteroduplex resistance will contribute to sexual isolation.

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