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Gene Exchange and Natural Selection Cause *Bacillus subtilis* to Evolve in Soil Culture

Abstract. *Strains of* Bacillus subtilis *exchange linked blocks of genes when growing together in soil; such exchange leads to extensive reorganization of the genotypic structure of the population and to the appearance and eventual dominance of a single phenotype. This process illustrates how recombination and selection lead to adaptive changes in populations.*

The controversy over the escape of bacteria carrying cloned genes and the concern over the spread of antibiotic resistance determinants among bacterial pathogens have focused chiefly on elements of human danger, real or putative. Most experiments demonstrating gene exchange between bacterial strains in their native habitats have involved human commensals or pathogens carrying antibiotic resistance determinants. Among these studies are those of Anderson *et al.* on plasmid transfer by conjugation of *Escherichia coli* strains in the human intestine (*1*); those of Novick and Morse on plasmid transfer by transduction of *Staphylococcus aureus* in the mouse kidney (*2*); those of Ottolenghi and MacLeod (*3*) and of Conant and Sawyer on transformation of pneumococcal strains in mixed peritoneal infections in mice (*4*); and those of Conant and Sawyer on transformation of pneumococcal strains in mixed respiratory infections in mice (*4*). Much of the controversy surrounding recombinant DNA is due to lack of knowledge of the natural lives of bacteria.

The anthropocentric emphasis, while important, has tended to obscure the fact that a quite general process of ecological and evolutionary adaptation is involved, its key feature being the generation by recombination of genetic variability in populations acted upon by selection. Hence, the question: If the parasexual processes of bacteria occur between organisms in their natural habitat, can they promote the acquisition of adaptive traits by bacterial populations? Our data show that for the soil bacterium *Bacillus subtilis*, the answer is yes. Genetic exchange is demonstrated by the transfer of linked blocks of chromosomal markers between strains growing together in soil culture; the ability of the exchange to promote adaptation is shown by the formation, spread, and eventual dominance in the cultures of a strain bearing markers from both parents. Here we present a complete analysis of the genetic results and consider them from an explicitly evolutionary point of view; the experimental details and supporting results have been described (*5*).

The strains used are called "168 R" and "SB-/R" and were constructed so that each carries a block of three linked genes and one outside marker unlinked to the block. Strain 168 R carries linked resistances to the antibiotics rifampicin (phenotypically, Rfm^r), erythromycin (Ery^r), and spectinomycin (Spc^r); the outside marker is lincomycin resistance (Lin^r). Strain SB-/R carries 3-aminotyrosine resistance (Amt^r) and histidine (His^-) and tryptophan (Trp^-) auxotrophic requirements as its linkage block and resistance to 4-azaleucine (Azl^r) as its outside marker. The two linkage blocks are opposite one another on the circular *B. subtilis* genetic map (*6*). This arrangement of markers permits the identification of triple transformants—the criterion for gene exchange—from both possible crosses by initially screening simultaneously for the outside marker and one marker in the linkage block of the recipient and one marker in the linkage block of the donor and by subsequent testing of the survivors for cotransfer of the other two markers in the donor linkage block. (We later discovered that Lin^r was linked to the Rfm^r Spc^r Ery^r block in soil transformations; however, the outcome of these experiments is not materially affected.)

Table 1. Occurrence of genetic exchange. All data are mean values of three mixed cultures.

Day	Triple transformants (%)	
	168 R × SB-/R*	SB-/R × 168 R†
1	0.02	0.02
2	0.005	0.02
3	0.003	0.005
4	0.0004	0.0003
6	0.000003	0.001
8	Not found	0.002

*Recipient × donor; triple transformants defined as Spc^r Lin^r Amt^r His^- Trp^-. †Recipient × donor; triple transformants defined as Amt^r Azl^r Spc^r Ery^r Rfm^r.

Spores of strains 168 R and SB-/R were separately treated with excess deoxyribonuclease and then heat-shocked (80°C; 15 minutes); 1×10^8 treated spores of each strain were then added to 50 g of sterile soil in a peat pot enclosed in a storage jar, along with 4 mg each of histidine and tryptophan; incubation was at 37°C. Samples were taken, suspended, diluted, and plated on selective minimal media or nonselective tryptose blood agar. Colonies arising on selective media were tested for cotransfer of linked markers (Table 1); colonies arising on nonselective media were tested for all markers (Table 2). These experiments take advantage of the facts that germinating spores both release transforming DNA (*7*) and become competent for transformation (*8*).

The rates of occurrence of triple transformants in mixed soil culture are shown in Table 1 for both possible crosses. These frequencies are at their greatest 10- to 100-fold lower than those obtained in laboratory transformation in liquid culture with saturating DNA, and they decrease with time. However, the frequencies are much larger than those that could result from three simultaneous mutations, about 10^{-18}. Higher frequencies, up to 17 percent, of triple transformants are observed when large amounts of phenol-extracted DNA (*9*) are added to strains growing alone in soil.

We have assumed that the demonstrated gene exchange in mixed soil culture is mediated by transformation since *B. subtilis* is not known to conjugate and since there are no known generalized transducing phages in these strains. However, we have not been able to block the exchange by the conventional laboratory methods of adding either deoxyribonuclease or competing DNA to the soil cultures.

The mixed cultures reached significantly higher stationary phase population sizes (2×10^8 to 4×10^8 colony-forming units per gram) than single-strain cultures either with or without

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added DNA [2×10^7 to 4×10^7 colony-forming units per gram; $P < .05$, Fisher exact probability test (*10*)]. This observation, coupled with the decrease in number of triple transformants mentioned above, suggested that there might be selection in the soil cultures against some of the markers. Testing of unselected colonies for all eight markers independently showed that the Spc^r, Azl^r, and Amt^r characters decreased in frequency to 15 percent or less and that the Rfm^r, Ery^r, Lin^r, His^-, and Trp^- characters increased to 90 percent or more. We cannot explain why any particular wild-type or mutant marker was favored, nor can we eliminate the possibility that the observed gene frequency changes were due to selection at loci linked to the ones actually tested. Similar testing of single-strain control cultures without added DNA revealed no changes from the parental phenotypes; hence the markers are stable.

When the complete phenotypes of all the colonies in each sample were coded, sorted, counted, and ordered by frequency by computer, it was seen that a single phenotype came to predominate in the mixed cultures (79 percent of all tested colonies on day 8) and that it had the combination of characters expected from the gene frequency changes: Spc^s Rfm^r Ery^r Lin^r Azl^s Amt^s His^- Trp^-. Moreover, the parental types disappeared almost immediately; SB-/R was not found in any sample, and 168 R was only present as two colonies out of 300 tested on day 1. Of the 256 possible combinations of the eight marker genes, 149 were found, even though we tested less than a millionth of the colony-forming units actually in the samples. The time course of the total number of distinct genotypes (Table 2) demonstrates both the generation of this large field of gene combinations by recombination and the subsequent sorting out and elimination of many of them by selection.

Some details of the selective process are given by the averages in Table 2. The trends discussed below, particularly the degree of consistency of events in the three replicates, can be seen fully only by inspecting the data for each replicate separately. Whereas the general trends can be readily detected in Table 2, the specific figures given below cannot be calculated from those data.

On day 1, the data reveal many phenotypes present at low frequencies (≤ 4 percent), an average of 44 phenotypes per replicate, and most phenotypes of higher frequencies (4 to 9 percent) detectable at those frequencies in only one replicate. Hence each replicate began its evolutionary course with a distinct array of phenotypes. The five most common phenotypes in each sample accounted for 27 to 47 percent of the total population.

By day 3 the early variability had been reduced; among the five most frequent phenotypes in each replicate, two types were present at 4 to 10 percent in all three populations, and three more were shared by two populations at 11 to 16 percent.

On day 4, three of the five most common phenotypes were shared by all three populations; the final most common phenotype, Spc^s Rfm^r Ery^r Lin^r Azl^s Amt^s His^- Trp^-, had frequencies of 26, 28, and 65 percent. The other two shared types ranged from 4 to 21 percent. The five most common phenotypes accounted for 80 to 90 percent of each population; the average number of types per replicate was 15. Moreover, in each replicate the five most common phenotypes other than the final most common one differed from it by only one or two markers. Thus, the three different starting arrays showed strong convergence to a few closely related types at higher frequencies.

By day 8, all three replicates had the final most common phenotype in high frequency (74, 80, and 83 percent). Once this phenotype was dominant in each replicate, the individual frequencies of all other phenotypes dropped to 5 percent or less. These remaining phenotypes do not closely resemble the final most common one.

Thus, the data show for a soil bacterium two major aspects of the evolutionary process, namely, the generation of genetic variability by recombination combined with the culling action of natural selection over time to produce adaptive change. The details for the five most common phenotypes (Table 2) offer an explicit empirical realization of the way in which a population is forced to explore the adaptive landscape (*11*) created jointly by its genes and its environment.

Table 2. Population dynamics of recombination and selection.

Day	Distinct phenotypes (No.)*	Five most common phenotypes†								
		Percent	Character‡							
			Spc	Ery	Rfm	Lin	Azl	Amt	His	Trp
1	92	9.4	s	r	s	r	s	s	−	−
		5.3	s	r	s	s	s	s	−	−
		3.3	r	r	r	r	s	r	−	−
		3.0	r	r	r	r	s	r	+	+
		2.7	s	r	s	s	s	s	+	+
2	81	10.3	r	r	s	s	s	s	−	−
		9.7	s	r	s	r	s	s	−	−
		7.0	s	r	s	s	s	s	−	−
		5.0	r	r	r	r	s	s	−	−
		3.7	r	r	r	r	s	r	+	+
3	48	30.0§	s	r	r	r	s	s	−	−
		9.4	r	r	r	r	s	s	−	−
		9.0	s	r	r	r	r	r	+	+
		8.7	r	r	r	r	r	r	+	+
		5.7	s	r	s	r	s	s	−	−
4	30	40.0§	s	r	r	r	s	s	−	−
		17.0	s	r	s	s	s	s	−	−
		13.3	s	r	r	s	s	s	−	−
		7.0	s	s	r	r	s	s	−	−
		5.7	s	r	s	r	s	s	−	−
6	33	45.0§	s	r	r	r	s	s	−	−
		15.7	s	r	r	s	s	s	−	−
		4.0	s	r	s	r	s	s	−	−
		3.7	s	r	r	r	s	r	−	−
		3.7	s	s	s	s	s	s	−	−
8	27	79.0§	s	r	r	r	s	s	−	−
		4.3	r	r	r	r	s	s	−	−
		3.7	r	s	r	s	s	r	−	−
		2.0	s	r	r	r	s	r	−	−
		1.0	s	r	s	s	s	r	+	−

*Data are combined from three replicate mixed cultures. †Data are mean values of three replicate mixed cultures. ‡Key: s, sensitivity to an agent; r, resistance; +, growth independent of an amino acid; −, requirement for an amino acid. §Final most common phenotype: Spc^s Rfm^r Ery^r Lin^r Azl^s Amt^s His^- Trp^-.

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Tectorial Membrane: A Possible Effect on Frequency Analysis in the Cochlea

Abstract. *Mathematical analysis and computer and network simulations of the cochlea show that, given appropriate values of specific physical constants, radial shear motion between the tectorial membrane and the reticular lamina may provide the sharpening of frequency analysis observed in cochlear nerve fibers in comparison with the mechanical amplitude distribution on the basilar membrane. According to the analysis, the sharpening occurs through an interaction of the longitudinal mechanical propagation constant of the tectorial membrane with the wavelength on the basilar membrane.*

On the basis of model investigations, Békésy (*1*) suggested that a coarse mechanical frequency analysis in the cochlea of the inner ear is sharpened by neural processes. More recent experiments confirm that neural tuning curves derived from the responses of cochlear nerve fibers are considerably sharper than corresponding mechanical filter curves measured on the basilar membrane (BM) of the cochlea (*2, 3*). Békésy's hypothesis in connection with these and related findings induced one of us (*4*) to suggest a specific neural sharpening mechanism. However, the suggestion has been invalidated by recent recordings from cochlear inner hair cells, which revealed that their receptor potentials as well as their impedance changes are as sharply tuned as the nerve fiber responses (*5*). This seems to restrict the possible sharpening mechanisms to mechanical ones acting either directly on the inner hair cells or distally to them. Although some such mechanisms have been suggested in the past (*6, 7, 8*), they are either difficult to reconcile with the currently available empirical information, are based on misconceptions, or lack plausible physical implementations. As a consequence, we attempted to look again at cochlear micromechanics. Somewhat unexpectedly, we saw a potential mechanism so obvious after the fact that it is difficult to understand how it could have remained undiscovered until now.

It is generally accepted that cochlear hair cells, like similar mechanoreceptors of the vestibular and lateral line systems, are excited by the deflection of their stereocilia toward their kinocilia or basal bodies (*9*). In the cochlea, this means a deflection in an approximately radial direction, away from the spiral bony lamina. In view of the observed mode of BM motion (*10*) and the structural relationships within the scala media, such a deflection is expected to occur during BM displacement toward the scala vestibuli (*11*). For the outer hair cells, the deflection must result from radial shear motion between the reticular lamina (RL), in which the cells are embedded, and the tectorial membrane (TM) to which their stereocilia are attached (*12*). Evidence for a similar attachment of the stereocilia of the inner hair cells is controversial but does not appear to be essential since the endolymph in the narrow gap between TM and RL must participate in the shear motion and entrain the stereocilia. In this respect it should be noted that the stereocilia are interconnected (*13*) so that the endolymph should not be able to move freely among them. Whether maximum excitation of the inner hair cells occurs during maximum BM velocity or displacement (*14, 15, 16*) is not crucial for the following analysis, however.

If the excitation of the hair cells is proportional to radial shear motion between the RL and TM, it must follow essentially the same distribution along the cochlea as the shear motion does. The latter must depend on the radial components of the mechanical coupling of the part of the TM overlying the hair cells to the organ of Corti and to the spiral limbus. If this part were tightly coupled to the organ of Corti and loosely to the spiral limbus, for instance, it would move together with the RL and no shear motion would occur. We show that, in addition, the shear motion must depend on the longitudinal coupling within the TM and on the wavelength on the BM. The situation is illustrated in Fig. 1. In agreement with anatomical evidence, the TM is assumed to be attached to the organ of Corti, most strongly at its outer margin and at the three or four rows of the outer hair cells and more weakly at the Hensen's stripe and the inner hair cells (*17, 18*). Near the basal end of the cochlea, the wavelength is the longest, and the phase of BM motion changes slowly with distance. Accordingly, the TM is driven in the same radial direction over a substantial length and should be maximally entrained. Thereby the radial shear motion between the TM and the RL should be minimized. As the wavelength becomes shorter toward the vibration maximum of the BM, the radial force on the TM rapidly changes direction with distance, and its effect should be partially nullified by the averaging effect of the longitudinal coupling within the TM. This averaging effect should minimize the radial TM motion and maximize the radial shear motion. The mechanism of enhancing the shear motion in regions of short wavelengths can be approximated analytically in terms of the mechanical analog shown in Fig. 1B. The longitudinal coupling in the TM per unit length is represented by the mechanical admittance $\mathbf{Y}_1$; $\mathbf{Z}_2$ is the mechanical impedance per unit length of the attachment to the organ of Corti, and $\mathbf{Z}_3$ the impedance of the corresponding coupling to the spiral limbus. $\mathbf{Y}_1$, $\mathbf{Z}_2$, and $\mathbf{Z}_3$ are assumed to apply to the radial components of motion of the TM and RL. Morphological appearance as well as static measurements (*10*) suggest that, outside the compliance of the BM, the mechanical elements are either constant throughout the cochlear length or vary slowly by comparison with the longitudinal space constant of the TM, so that their longitudinal space derivatives can be neglected. Since the purpose of this report is to demonstrate a principle rather than to describe the exact conditions in the cochlea, we assume for simplicity that the mass of the TM is negligible and that all the impedances, $1/\mathbf{Y}_1$, $\mathbf{Z}_2$, and $\mathbf{Z}_3$, reflect similar viscoelastic

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