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Creating New Genes by Plasmid Recombination in *Escherichia coli* and *Bacillus subtilis*

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Gene shuffling is a way of creating proteins with interesting new characteristics, starting from diverged sequences. We tested an alternative to gene shuffling based on plasmid recombination and found that *Bacillus subtilis* efficiently recombines sequences with 4% divergence, and *Escherichia coli mutS* is more appropriate for sequences with 22% divergence.

The technique of gene shuffling is presently essentially based on PCR (10, 12), which creates an enormous waste of useless sequences containing deletions or nonsense mutations. Much less waste is expected in vivo, since the recombination proteins are designed to promote in-register exchanges, without deletions, and since the rate of mutagenesis is lower than that of the PCR polymerases. We therefore decided to test the efficiency of creating new genes by plasmid recombination in vivo.

Experiments were conducted with two distantly related bacterial hosts, *Escherichia coli* and *Bacillus subtilis*, in which the mechanisms of homologous recombination are well known. Recombination efficiencies were measured as a function of DNA divergence (0%, 4%, and 22% divergence) and plasmid vector type (symmetrical, theta replication versus asymmetrical, rolling-circle replication). The recombination substrates were three 800-bp-long OXA genes, the OXA-7, OXA-11, and OXA-5 genes, encoding related beta-lactamases differing from the OXA-7 beta-lactamase at the nucleotide level by 0%, 4%, and 22%, respectively. They were cloned into two plasmids: pACYC184, a plasmid of *E. coli* that cannot replicate in *B. subtilis*, and pIL253, a plasmid of *B. subtilis* that cannot replicate in *E. coli*. In each bacterial host, experiments were conducted so as to maximize recombination efficiency. For *E. coli* electrotransformations, DNA was UV irradiated at a dose of 200 J/m^2 so that the yield of recombinants was 10-fold higher than that of nonirradiated DNA. Transformation in *B. subtilis* is a natural process, involving the cutting of double-strand DNA and the degradation of one strand, so that DNA enters as single-strand linear DNA. In both species, recipient strains for the transformation experiments harbored a replicative plasmid sharing no significant stretch of identity with the incoming DNA, except for the presence of an OXA gene, so that the establishment of the incoming DNA is dependent on its integration into the OXA gene of the resident plasmid. The selection of clones harboring recombinant plasmids was based on a marker(s) encoded by the nonreplicative plasmid (Spc^r Phl^r in *E. coli* and Erm^r in *B. subtilis*). An estimation of the recombination frequency was obtained by dividing the transformation efficiency of the nonreplicating plasmid by the transformation efficiency of a control, replicative plasmid. *E. coli* cells had a much higher competence (between 10^6 and 10^8 transformants per microgram of control DNA) than the *B. subtilis* cells (0.5×10^5 to 1.5×10^5 transformants per microgram of control DNA). For the *B. subtilis* experiments, two different recipient vectors were tested: a theta-replicating vector, pMAP176, in which replication of the two strands is simultaneous so that

TABLE 1. Shuffling efficiencies

% Divergence	Recombination efficiency in indicated genetic background[a]					
	E. coli theta plasmid		*B. subtilis* theta plasmid		*B. subtilis* RC plasmid	
	WT	*mutS*	WT	*mutLS*	WT	*mutLS*
0	3.6×10^{-4}	9.3×10^{-4}	2.0×10^{-3}	4.2×10^{-3}	1.5×10^{-1}	6.4×10^{-2}
4	3.1×10^{-8}	2.0×10^{-5}	2.5×10^{-4}	9.8×10^{-3}	3.0×10^{-1}	7.0×10^{-2}
22	$<5.5 \times 10^{-8}$	2.6×10^{-7}	$<2.5 \times 10^{-4}$	$<2.5 \times 10^{-4}$	$<1.4 \times 10^{-4}$	$<1.4 \times 10^{-3}$

[a] RC, rolling circle; WT, wild type.

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TABLE 2. Recombinant-product analysis

Parameter	*E. coli* theta plasmid	*B. subtilis* theta plasmid	*B. subtilis* RC plasmid[a]
No. of OXA genes sequenced	108	22	22
No. of novel genes (%)	58 (53)	11 (50)	8 (36)
No. of novel proteins (%)	38 (35)	8 (36)	7 (32)
% of products:			
Reciprocal	20	0	0
Nonreciprocal	50	82	64
Mosaïc	30	18	18
Gene conversion	0	0	18

[a] RC, rolling circle.

little single-strand DNA and no double-strand ends are present (2), and a rolling-circle-replicating vector, pMAP183, which replicates its two strands successively so that double-strand ends and single-strand DNA are present (11). The theta-replicating vectors of *E. coli* and *B. subtilis* had similar replication cycles and copy numbers (~5). Results are shown in Table 1.

In wild-type *E. coli*, the recombination frequency between identical sequences was 3.6×10^{-4}. A 4% DNA divergence reduced recombination by 10,000-fold in the wild type, but inactivation of the *mutS* gene had the expected effect of increasing the recombination efficiency by 1,000-fold (7) so that the net yield of transformants was 4,000 transformants per microgram of DNA. No transformants were recovered from the wild-type strain upon the selection of recombinants with 22%-divergent sequences, but in the *mutS* background, 40 transformants per microgram of DNA were obtained. The frequency of recombination (2×10^{-7}) was lower than the rate of spontaneous Phl^r Spc^r mutations arising in *E. coli mutS* (5×10^{-6}), so that the plasmid content of ~100 clones was analyzed to detect these recombinations.

In the wild-type *B. subtilis* host, the recombination frequency

TABLE 3. Strains and plasmids

Name	Genotype	Source/construction
E. coli strains		
AB1157 Nal	Like AB1157 but Nal^r	M. Radman strain collection
MIXP1	*mutS*::Tn*5* (*Kan*^{r})	P1 transduction of *mutS*::Tn*5* into AB1157 Nal
Plasmids		
pHV1210	pE194-pBR322 hybrid	Noirot et al. (6)
pIC156	pUC1318-Spc^r	Steinmetz and Richter (9)
pUC-Phleo	pUC19-Phl^r	E. Dervyn collection
pMIX92	pUC19-Spc^r-Phl^r	Integration of the Spc^r cassette of pIC156 (BamHI EcoRI) into pUC19-Phl^r
pMIX93	pACYC184-OXA-7	Integration of OXA-7 or *E. coli* into pACYC184 (ScaI-PpuMI)
pMIX95	pACYC184-OXA-11	Integration of OXA-11 of *P. aeruginosa* into pACYC184 (BamHI-EcoRI)
pMIX96	pACYC184-OXA-5	Integration of OXA-5 of *P. aeruginosa* plasmid pMON812 into pACYC184 BamHI-EcoO109I
pMAP177	pACYC184-Erm^r	Integration of the Erm^r cassette of pHV1210 (ClaI SacI) into pACYC184 (ClaI-FspI)
pMAP178	pACYC184-Erm^r-OXA-7	Integration of the Erm^r cassette of pHV1210 (ClaI SacI) into pMIX93 (ClaI SacI)
pMAP179	pACYC184-Erm^r-OXA-11	Integration of the Erm^r cassette of pHV1210 (ClaI SacI) into pMIX95 (ClaI-FspI)
pMAP180	pACYC184-Erm^r-OXA-5	Integration of the Erm^r cassette of pHV1210 (ClaI SacI) into pMIX96 (ClaI-FspI)
B. subtilis strains		
PB1856	Δ*mutLS*	Ginetti et al. (3)
SB202	WT[a]	
MAS 831	Δ*mutLS*	Transformation of the *mutLS*::Cm^r allele from strain PB1856 into SB202
Plasmids		
pIL253		Simon and Chopin (8)
pIL252		Simon and Chopin (8)
pMIX90	pIL253-Spc^r	Integration of the Spc^r cassette of pIC156 (SacI) into pIL253
pMIX91	pIL253-Spc^r-Phl^r	Integration of the Phl^r cassette of pUC19-Phlco (EcoRI SalI) into pMIX90
pMIX94	pMIX91-OXA-7	Integration of OXA-7 from pMIX93 into pMIX91
pMIX98	pMIX91-OXA-11	Integration of OXA-11 from pMIX95 into pMIX91
pMAP176	pIL252-Spc^r-OXA-7	Integration of the Spc^r-OXA-7 cassette of pTG2 into pIL252 (EcoRI-Eco1091 sites)
pMAP183	pUB110-OXA-7	Integration of the OXA-7 cassette of pMAP178 (PstI-NruI) into the PvuII site of pUB110 (orientation such that OXA-7 and the Kan^r gene are convergent)

[a] WT, wild type.

between identical sequences was 2×10^{-3} on theta plasmids and 100-fold more efficient on the rolling-circle vector. This confirms earlier reports showing that rolling-circle plasmids are hyperrecombinogenic (4). Moderate divergence (4%) decreased recombination in wild-type *B. subtilis* by 10-fold only (in the theta plasmid) or not at all (in the rolling-circle plasmid). Indeed, moderate divergence is much more "tolerated" in wild-type *B. subtilis* than in wild-type *E. coli* (5, 7). The Δ*mutLS* mutation increased recombination efficiency in the *B. subtilis* theta plasmid by a factor of 40. No recombinants were detected for sequences with a divergence of 22% (frequency below 10^{-4}). It has been shown previously that in *B. subtilis*, recombination frequencies remained high, with sequences having up to 7% divergence, but then decreased sharply irrespective of the presence or absence of MutL and MutS (5). We speculate, therefore, that 22%-divergent sequences are unlikely to recombine in *B. subtilis*.

Chimeric genes resulting from recombination between the 4%-divergent OXA genes were sequenced in 54 plasmids of *E. coli* and 22 plasmids of *B. subtilis* (11 for each plasmid type). Results are reported in Table 2. According to classical crossing-over models, the two DNA strands of a recombined plasmid are expected to be slightly different. Depending on which strand will give a progeny, different products may thus be recovered. "Reciprocal products" are expected if the progeny derives from the strand cut by the RuvABC resolvase (for *E. coli*) or by RuvAB and probably RecU (for *B. subtilis*) (1). However, if the other strand is kept, and depending on its processing, "nonreciprocal products" could be observed. More-complex situations can be encountered, such as multiple crossovers giving "mosaic" products and "gene conversion" products, where a gene conversion process precedes the crossover step. Under all conditions tested, the main category was "nonreciprocal" products (50 to 82% of the recombinants). Mosaic products were also found in all cases (18 to 30%). Interestingly, reciprocal products were recovered only in *E. coli*. Conversely, gene conversion products were found only in *B. subtilis*, with the rolling-circle plasmid vector.

All strains and plasmids used in this study are described in Table 3.

We conclude that plasmid vectors are appropriate tools to create new genes by recombination, with *B. subtilis* being a slightly better host for a low level of divergence (4%) and the *E. coli mutS* strain being more appropriate for a high level of divergence (up to 22%). This in vivo technique should therefore apply to attempts at creating new gene combinations starting from ortholog genes of related species, or paralogs within a species, so that the divergence at the DNA level is less than 20%. It does not apply, however, to the creation of hybrid genes composed of a combination of two unrelated sequences. Nevertheless, we predict that in vivo recombination should also be possible when the starting sequences are composed of highly conserved regions interspersed with regions of high divergence.

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