labelled h-SIE probe (5′-CATTTCCCGTAAATC-3′)[18] containing a protruding poly-G tail at both sense and antisense 5′ ends. HGF-induced stimulation of transcriptional activities of the isolated c-*fos* (ref. 26) and *waf-1* (ref. 27) promoters was measured by the Luciferase assay[23]. Synthesis of $p62^{c\text{-}fos}$ and $p21^{waf\text{-}1}$ was measured by western blot with specific antibodies (gifts from C. Schneider). Cell 'scattering' in response to HGF (50 ng ml^{-1}) was measured as described[1]. Micrographs (×1,000) were taken 6 h after stimulation. Stimulation of growth was assessed by ^{3}H-thymidine uptake 12 h after HGF stimulation of cells synchronized before electroporation by two cycles of thymidine (2 mM)-deoxycitidine (250 μM). Formation of branched tubular structures was monitored on cell monolayers, coated with a 0.5-mm layer of collagen G (2 mg ml^{-1}) in the presence of HGF (10 ng ml^{-1}) supplemented daily[1].

Phosphopeptides, decoys and electroporation. N-acetylated and C-amidated synthetic peptides, mimicking the consensus sequences for SH2-signal transducers in the HGF receptor or in the STAT family, were synthesized in their phosphorylated or non-phosphorylated forms by Fmoc chemistry, and purified by high-performance liquid chromatography (HPLC; >99%), as will be described elsewhere. For *in vivo* electroporation the phosphate group was substituted by the non-hydrolysable methylenphosphonate group. The h-SIE decoy (5′-CATTTCCCGTAAATC-3′) and its scrambled control 5′-ACTCTTGCCAATTAC-3′ were synthesized and used in the inhibition assay as described[28]. For electroporation, adherent cells were cultured onto 1-cm^2 indium-tin oxide conductive glass surfaces and electroporated *in situ* with a PBS solution containing either the competing peptides (1 μM) or the decoys (10 μM). The electric pulse was generated using the Epizap apparatus (Ask Science, Canada), following the technique described previously[29]. More than 90% of cells were permeabilized as tested by the uptake of Lucifer yellow, and viability was higher than 80% as judged by trypan blue exclusion.

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DNA shuffling of a family of genes from diverse species accelerates directed evolution

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DNA shuffling is a powerful process for directed evolution, which generates diversity by recombination[1,2], combining useful mutations from individual genes. Libraries of chimaeric genes can be generated by random fragmentation of a pool of related genes, followed by reassembly of the fragments in a self-priming polymerase reaction. Template switching causes crossovers in areas of sequence homology. Our previous studies used single genes and random point mutations as the source of diversity[3–6]. An alternative source of diversity is naturally occurring homologous genes, which provide 'functional diversity'. To evaluate whether natural diversity could accelerate the evolution process, we compared the efficiency of obtaining moxalactamase activity from four cephalosporinase genes evolved separately with that from a mixed pool of the four genes. A single cycle of shuffling yielded eightfold improvements from the four separately evolved genes, versus a 270- to 540-fold improvement from the four genes shuffled together, a 50-fold increase per cycle of shuffling. The best clone contained eight segments from three of the four genes as well as 33 amino-acid point mutations. Molecular breeding by shuffling can efficiently mix sequences from different species, unlike traditional breeding techniques. The power of family shuffling may arise from sparse sampling of a larger portion of sequence space.

Reiterative cycles of shuffling followed by screening or selection has proved to be a useful approach for the evolution of single gene products with enhanced activity[3], altered substrate specificity[4] or improved protein folding[5] and of entire operons with improved function[6]. When a single starting sequence is used, diversity originates as random point mutations resulting from the polymerase reaction[1]. Because most point mutations are deleterious or neutral[7], the random point mutation rate must be low[8] and the accumulation of beneficial mutations and the evolution of a desired function is relatively slow in such experiments. For example, the evolution of a fucosidase from a galactosidase required five rounds of shuffling and screening before a >10-fold improvement in activity was detected[4]. Naturally occurring homologous sequences are pre-enriched for 'functional diversity' because deleterious variants

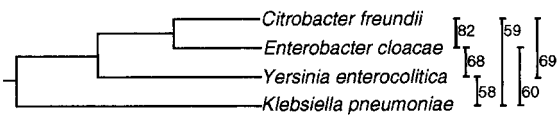

Figure 1 Phylogenetic tree of the four cephalosporinase genes. The numbers on the vertical bars indicate the percentage of DNA sequence similarity.

have been selected against over billions of years of evolution. We therefore wanted to determine whether shuffling of gene families would accelerate the evolution process.

Four 1.6 kilobase (kb) genes encoding class C cephalosporinases, 58–82% identical at the DNA sequence level (Fig. 1), were chosen from four microbial species (*Citrobacter freundii*[9], *Enterobacter cloacae*[10], *Klebsiella pneumoniae*[11] and *Yersinia enterocolitica*[12]) and constructed by gene synthesis[13] using the original DNA sequence. The four genes were shuffled either individually or as a pool using methods previously described[1,3] (Fig. 2a). Equal numbers of *Escherichia coli* transformants (about 5×10^4) expressing the resultant libraries were then plated on media containing a range of concentrations of the antibiotic moxalactam (0.016–32 $\mu g\,ml^{-1}$). After this single round of shuffling, the colonies showing the highest level of resistance to moxalactam were identified and further characterized.

Clones originating from the four single gene libraries showed up to eightfold increases in moxalactam resistance as compared to those expressing the wild-type genes (0.38 to 3.0 $\mu g\,ml^{-1}$ for the *Klebsiella* and *Yersinia* genes and 0.75 to 6.0 $\mu g\,ml^{-1}$ for the *Citrobacter* and *Enterobacter* genes). In contrast, the best clone (A) originating from the gene family library showed a 540-fold increase in resistance (0.38 to 200 $\mu g\,ml^{-1}$) compared to the wild-type *Klebsiella* and *Yersinia* genes and a 270-fold increase (0.75 to 200 $\mu g\,ml^{-1}$) compared to the wild-type *Enterobacter* and *Citrobacter* genes (Fig. 2a). Thus 'family shuffling' accelerated the rate of functional enzyme improvement 34- to 68-fold in a single cycle.

A second round of shuffling was performed using the pool of colonies selected in the first round. Plating of about 5×10^4 colonies on a range of concentrations of moxalactam yielded three clones which had a further 3.5-fold increased moxalactam resistance over the most resistant clone for the first cycle (clone A). The reduction in the rate of improvement is expected given the limited dynamic range of the bioassay. Based on our experience with other genes[3–6], a second round of intra-species shuffling of the selected pools containing the eightfold improved genes would clearly not have achieved the degree of improvement we obtained by two rounds of inter-species shuffling.

The two most resistant clones (A and B) obtained in the first round of family shuffling also showed increased resistance against other β-lactam antibiotics. Clone A was resistant to cefoxitine, carbenicillin and cephafloridine at 100 $\mu g\,ml^{-1}$ each, an improvement over the four wild-type enzymes of 4–16-fold. This was unexpected because in previous results for single gene shuffling of other enzymes the activity for the original substrate was decreased after evolution for increased activity on a new substrate[3,4]. Plasmid transfer experiments into *E. coli* NM522 showed that all of the increased resistance was conferred by the plasmid. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of periplasmic extracts showed that the expression level of the four wild-type cephalosporinases was indistinguishable from that of the mutant clones A and B (data not shown). Antibiotic bioassays showed that all of the moxalactam was degraded by cells expressing chimaeric enzyme A or B, but not by cells expressing any of the four wild-type enzymes. This indicates that the specific activity of the chimaeric moxalactamases was improved. However, our attempts to obtain and compare the kinetic profiles of the wild-type and chimaeric enzymes failed because of the very low activity of the wild-type enzymes.

The genes encoding the new moxalactamases from these two clones were sequenced. Clones A and B were both found to be chimaeras of the genes from *Citrobacter*, *Enterobacter* and *Klebsiella*. Both clones had a similar overall structure containing eight segments resulting from seven crossovers. Because of local DNA homology the crossover location could not be defined more exactly than indicated by the grey segments shown in Fig. 2b. The crossovers occurred in areas where the two genes had 14–37 base pairs (bp) of nearly identical sequence (average 57% GC). In addition, chimaera A had 47 DNA point mutations, 6 of which were silent, resulting in 33 amino-acid substitutions scattered throughout the gene that did not exist in any of the four parental enzymes.

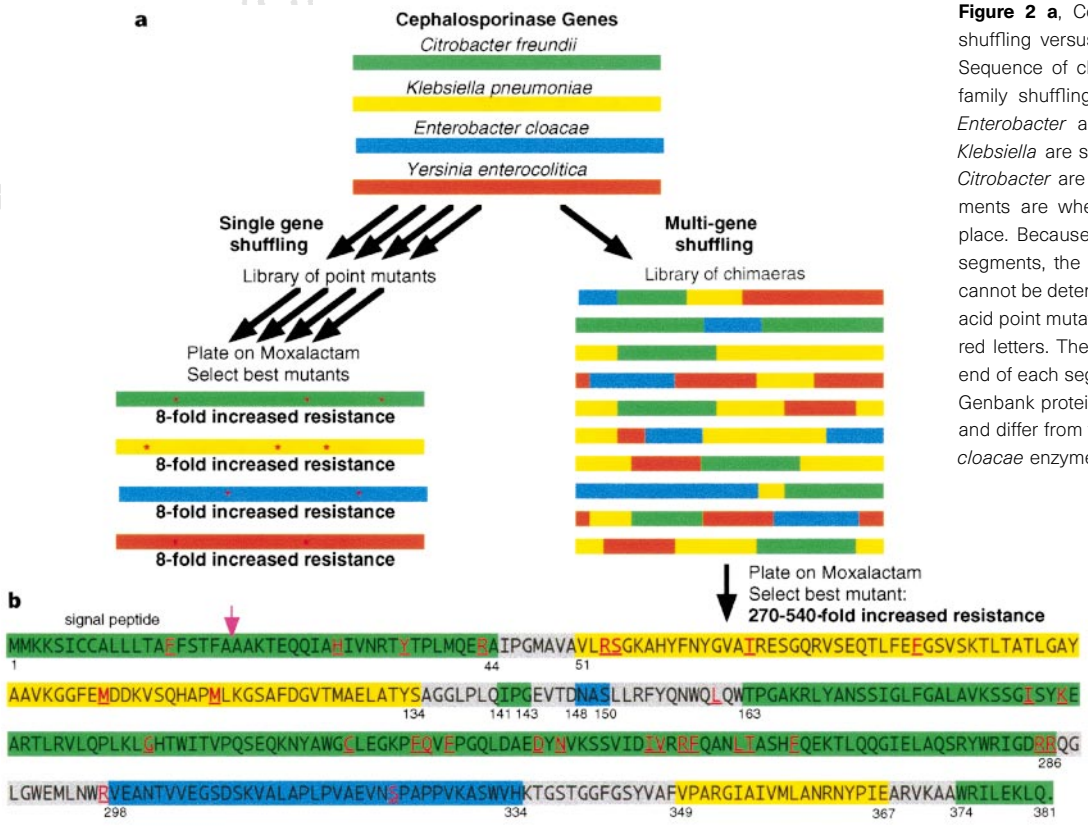

Figure 2 **a**, Comparison of single sequence shuffling versus sequence family shuffling. **b**, Sequence of chimaeric mutant A obtained by family shuffling. The segments derived from *Enterobacter* are shown in blue, those from *Klebsiella* are shown in yellow, and those from *Citrobacter* are shown in green. The grey segments are where the crossovers have taken place. Because of DNA homology in the grey segments, the exact location of the crossover cannot be determined more exactly. The amino-acid point mutations are shown with underlined red letters. The numbers at the beginning and end of each segment are the numbers from the Genbank protein files of the wild-type enzymes and differ from those used for the *Enterobacter cloacae* enzyme[14].

Chimaera B had 14 amino-acid substitutions, of which 12 were identical to those in chimaera A.

A model of the best chimaeric moxalactamase, chimaera A, was created from the known structure of the *Enterobacter cloacae AmpC* enzyme[14] (Fig. 3). Although 37% of the amino acids (142 residues) of the chimaeric clone A differ from the *Enterobacter cloacae* enzyme for which the crystal structure is known, after energy minimization the predicted structure of the α-chain backbone of the A chimaera remained nearly identical to the known structure (r.m.d.s. deviation of 0.766 Å). This model shows that two crossovers occurred in loop or random coil regions separating α-helical and β-sheet structures, and one occurred inside the C-terminal α-helix. The remaining five crossovers could have occurred in loops, α-helices or β-sheets; therefore it is unclear whether the crossovers preferentially occurred in loops separating structural elements. The conserved catalytic residues S64, K67, Y150 and K315 of the *Enterobacter* enzyme[14] were retained.

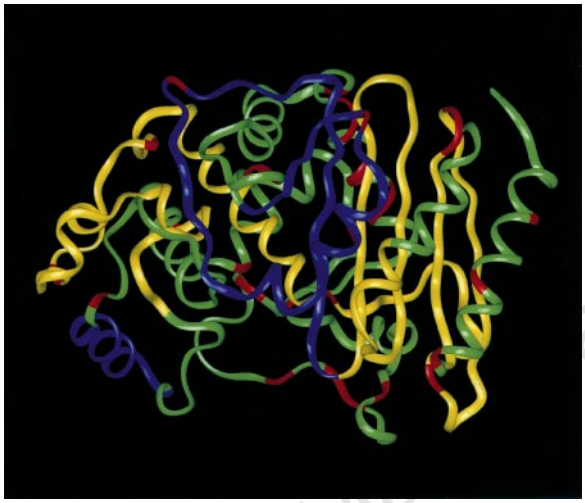

Figure 3 Computer model of evolved mutant A obtained by a single cycle of family shuffling. The 142 amino-acid mutations were introduced into the *Enterobacter cloacae* sequence, whose structure is known[14], followed by energy minimization. The predicted structure of the α-chain backbone of the chimaeric enzyme is within an r.m.s. deviation of 0.766 Å from the known native structure. The segments derived from *Enterobacter* are shown in blue, those from *Klebsiella* are shown in yellow, and those from *Citrobacter* are shown in green. The 33 amino-acid point mutations are shown in red.

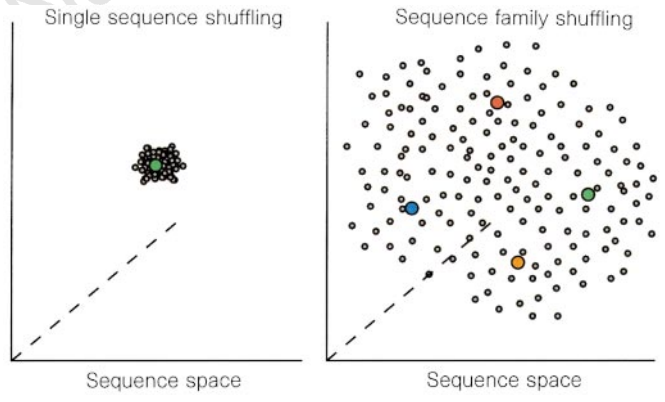

Figure 4 Searching sequence space by family shuffling versus by single sequence shuffling. Single sequence shuffling yields clones with a few point mutations and the library members are typically 97–99% identical. Family shuffling causes sequence block exchange which yields chimaeras that have greater sequence divergence. At equal library size, the increased sequence diversity of the chimaeric library results in sparse sampling of a much greater area of sequence space, allowing more promising areas to be found and subsequently explored at increased sampling density.

Although shuffling of a single gene creates a library of genes that differ by only a few point mutations[1–6], the block-exchange nature of family shuffling creates chimaeras that differ in many positions. For example, in previous work a single β-lactamase gene was shuffled for three cycles, yielding only four amino-acid mutations[3], whereas a single cycle of family shuffling of the four cephalosporinases resulted in a mutant enzyme which differs by 102 amino acids from the *Citrobacter* enzyme, by 142 amino acids from the *Enterobacter* enzyme, by 181 amino acid from the *Klebsiella* enzyme and by 196 amino acids from the *Yersinia* enzyme. The increased sequence diversity of the library members obtained by family shuffling results in a 'sparse sampling' of a much greater portion of sequence space[15], the theoretical collection of all possible sequences of equal length, ordered by similarity (Fig. 4). Selection from 'sparse libraries' allows rapid identification of the most promising areas within an extended sequence landscape (a multidimensional graph of sequence space versus function)[15]. However, the sparseness also decreases the likelihood of immediate location of the area's best sequence, the local optimum. Subsequent shuffling of the selected sequences allows further exploration of the still vast intermittent sequence space at an increased sampling density. Although the search algorithm remains unchanged, the scale of the searched area decreases with each cycle until no further improvement occurs.

Because the species definition in eukaryotes is based on the ability to exchange genetic information, the family shuffling that occurs in natural evolution and in classical breeding is generally restricted to the diversity existing within a single species. DNA shuffling can successfully mix genes from diverse species because single genes can tolerate much higher mutation densities[16] than whole genomes. The high degree of DNA homology that is required for traditional breeding of plants and animals is therefore not required for the molecular breeding of single genes and gene clusters. □

Methods

Gene synthesis. All four 1.6 kb genes were separately assembled from 60-mer synthetic oligonucleotides in a single assembly reaction[13]. These genes were cloned into pBR322 downstream of the β-lactamase promoter and in place of the native β-lactamase gene. The DNA sequence and drug resistance of each construct was confirmed.

Library construction. The presence of chimaeric genes in the library made by family shuffling was confirmed by restriction fragment length polymorphism (RFLP) analysis of individual clones. Each of the eight clones analysed had an RFLP pattern distinct from each other as well as from those of the parental sequences. The library thus contained a diverse array of chimaeric sequences. The second cycle of shuffling was performed on the pool of chimaeras selected in the first round.

Drug resistance. An equal number of transformants were plated on medium containing moxalactam. The numbers were based on the number of tetracyclin-resistant colonies after transformation. The resistance of the selected clones to a variety of β-lactam antibiotics was measured in 96-well plates in which each antibiotic was serially diluted (1 : 1) into culture medium containing the test organism. Assays for all native and mutant clones were done in quadruplicate.

Moxalactamase assays. The moxalactam degradation activity of whole cells was measured by incubating an overnight culture of bacteria with 34 μg ml^{-1} of moxalactam in LB media. After incubation for 16 h the concentration of moxalactam in the sterilized supernatant was measured by bioassay on plates inoculated with *E. coli* NM522, using filter discs saturated with serial twofold dilutions of the supernatants. A moxalactam standard curve was used to determine the fraction of moxolactam degraded by the host cell alone, the four wild-type constructs and the two chimaeric mutants.

Modelling. The deduced amino-acid sequence of chimaeric mutant A was aligned with the wild-type *Enterobacter* sequence using HOMOLOGY (Biosym, San Diego) and the calculated coordinates were assigned to the chimaeric protein. The modelled structure was constructed using a three-step protocol that involved an energy minimization to relieve steric hindrance, molecular

dynamics calculations to find the lowest energy conformation followed by another energy minimization to provide the final structure.

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A bacterial antibiotic-resistance gene that complements the human multidrug-resistance P-glycoprotein gene

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Bacteria have developed many fascinating antibiotic-resistance mechanisms[1,2]. A protein in *Lactococcus lactis*, LmrA, mediates antibiotic resistance by extruding amphiphilic compounds from the inner leaflet of the cytoplasmic membrane[3,4]. Unlike other known bacterial multidrug-resistance proteins, LmrA is an ATP-binding cassette (ABC) transporter[5]. The human multidrug-resistance P-glycoprotein[6], encoded by the *MDR1* gene, is also an ABC transporter, overexpression of which is one of the principal causes of resistance of human cancers to chemotherapy[7,8]. We expressed *lmrA* in human lung fibroblast cells. Surprisingly, LmrA was targeted to the plasma membrane and conferred typical multidrug resistance on these human cells. The pharmacological characteristics of LmrA and P-glycoprotein-expressing lung fibroblasts were very similar, and the affinities of both proteins for vinblastine and magnesium-ATP were indistinguishable. Blockers of P-glycoprotein-mediated multidrug resistance also inhibited LmrA-dependent drug resistance. Kinetic analysis of drug dissociation from LmrA expressed in plasma membranes of insect cells revealed the presence of two allosterically linked drug-binding sites indistinguishable from those of P-glycoprotein. These findings have implications for the reversal of antibiotic resistance in pathogenic microorganisms. Taken together, they demonstrate that bacterial LmrA and human P-glycoprotein are functionally interchangeable and that this type of multidrug-resistance efflux pump is conserved from bacteria to man.

Using the polymerase chain reaction (PCR), the bacterial *lmrA* coding sequence[3] was cloned into a pCI-neo mammalian expression vector under the control of the human cytomegalovirus immediate–early enhancer/promoter region. A Kozak sequence[9] was introduced at the ATG initiation codon of *lmrA* to enhance translational efficiency. For control experiments, a transport-inactive LmrA protein was generated in the same vector by introducing a lysine-to-methionine substitution at position 388 (K388M)[10] in the Walker A motif of the nucleotide-binding domain of the protein by site-directed mutagenesis. Hexa-histidine tags were also added to the amino termini of both the wild-type and K388M forms of LmrA.

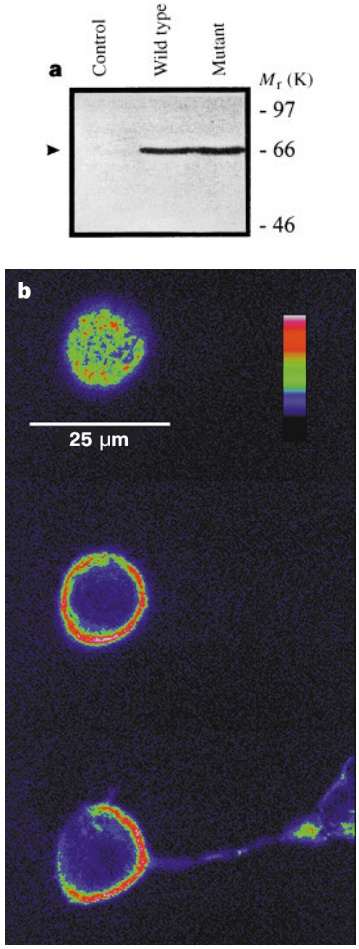

Figure 1 Expression of LmrA in GM0637 human lung fibroblast cells. **a**, Western blot of total cell protein (30 μg per lane) using the anti hexa-histidine tag antibody. Control, wild type and mutant refer to mock-transfected cells, and cells expressing the wild-type and K388M forms of LmrA, respectively. The migration of molecular mass markers is indicated. Arrowhead indicates LmrA protein. **b**, Distribution of wild-type LmrA expressed in fibroblast cells 48 h after transfection. The three frames show cross-sections through a cell, from top to bottom along the *z*-axis. The fluorescence intensity is shown colour-coded on a scale from blue (low) to white (high). Scale bar, 25 μm.