

Directed Evolution of Protein Inhibitors of DNA-nucleases by *in Vitro* Compartmentalization (IVC) and Nano-droplet Delivery

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In vitro compartmentalization (IVC) uses water-in-oil emulsions to create artificial cell-like compartments in which genes can be individually transcribed and translated. Here, we present a new application of IVC for the selection of DNA-nuclease inhibitors. We developed a nano-droplets delivery system that allows the transport of various solutes, including metal ions, into the emulsion droplets. This transport mechanism was used to regulate the activity of colicin nucleases that were co-compartmentalized with the genes, so that the nucleases were activated by nickel or cobalt ions only after the potential inhibitor genes have been translated. Thus, genes encoding nuclease inhibitors survived the digestion and were subsequently amplified and isolated. Selection is therefore directly for inhibition, and not for binding of the nuclease. The stringency of selection can be easily modulated to give high enrichments (100–500-fold) and recoveries. We demonstrated its utility by selecting libraries of the gene encoding the cognate inhibitor of colicin E9 (immunity protein 9, or Im9) for inhibition of another colicin (ColE7). The *in vitro* evolved inhibitors show significant inhibition of ColE7 both *in vitro* and *in vivo*. These Im9 variants carry mutations into residues that determine the selectivity of the natural counterpart (Im7) while completely retaining the residues that are conserved throughout the family of immunity protein inhibitors. The *in vitro* evolution process confirms earlier hypotheses regarding the “dual recognition” binding mechanism and the way in which new colicin-immunity pairs diverged from existing ones.

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Keywords: directed-evolution; emulsion; nano-droplets; colicin; immunity protein

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Introduction

There exist a number of high-throughput display selection strategies based on a physical linkage between the gene and the protein it encodes.^{1–5} These provide a powerful means of selecting proteins that bind any given ligand. However, the established rule of “you get what you select for”⁶ surmises that indirect selections are generally ineffective. Thus, selections of enzymatic activities *via* a binding step (e.g. to substrates or inhibitors) are less effective than a direct selection for high

turnover rates.^{7–11} Similarly, a selection for inhibitors by binding of the target enzyme may yield proteins or peptides that tightly bind the enzyme outside its active site, and are therefore poor inhibitors.^{12–14} In this context, a system based on *in vitro* compartmentalization (IVC) was developed, that provides a flexible mean of linking genotype to phenotype, which enables the selection not by binding only (as with other *in vitro* approaches) but also by enzymatic, regulatory and inhibitory activities. The basic concept is quite simple: a water-in-oil (w/o) emulsion is formed that offers $>10^{10}$ aqueous micro-droplets in 1 ml of oil. In these artificial cell-like compartments of approximately 2 μm diameter and a volume of about 5 fl, a variety of biochemical processes take place while the external oil phase remains inert. IVC was applied to select binding as well as enzymatic activities.^{15–22}

Abbreviations used: IVC, *in vitro* compartmentalization; Im, immunity; IPTG, isopropyl β -D thiogalactoside; w/o, water-in-oil.

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Here, we describe a new application of IVC in the direct selection of inhibitors of DNA-nucleases. A library of genes is compartmentalized, and single genes are allowed to transcribe and translate within aqueous droplets that also contain a DNA-nuclease. Thus, genes encoding a peptide or protein that inhibits the nuclease survive, whilst other genes, that do not encode an inhibitor, are digested. However, this strategy requires a regulatory mechanism that activates the nuclease only after gene translation has been completed. Or else, all genes would be indiscriminately digested before they had the chance to be translated. We therefore developed a delivery system based on the solubilization of water-soluble ions in nano-droplets (or swollen micelles) and the merging of these droplets with the aqueous droplets of the IVC emulsion, thus enabling us to regulate processes within the emulsion droplets after they were formed. We demonstrate this system in the selection of inhibitors for colicin DNases (ColEs) while taking advantage of the fact that bivalent metal ions such as nickel or cobalt, that can be delivered by nano-droplets, activate ColEs.^{23,24} The newly developed selection strategy is presented schematically in Figure 1.

We specifically chose to explore the colicin endonucleases and their natural inhibitors, the immunity proteins, as they comprise an interesting system of molecular synergism evolved by nature. Colicin endonucleases are used by *Escherichia coli* to kill competing bacterial strains under stress conditions. The immunity (Im) proteins provide protection to the attacking bacteria from destruction of their own DNA. Following the co-expression and secretion of the ColE-Im complex, the ColE is released from its Im inhibitor, and is free to attack other bacteria.^{25,26} There are four known pairs of DNase ColE-Im in *E. coli*, although many more

pairs probably exist in nature. These cognate pairs bind with extremely high affinity ($K_a \geq 10^{14} \text{ M}^{-1}$) and selectivity (binding of non-cognate partners is 10^6 – 10^{10} -fold weaker than cognate binding).

The *in vitro* selection system described here exhibits high enrichments and a wide dynamic range, as demonstrated in model selections of genes encoding a cognate *versus* a non-cognate immunity. Selection for the inhibitor is direct, genes are selected by virtue of their ability to encode a protein that inhibits the DNA-nuclease activity, rather than simply bind the ColE. We applied this system to reproduce the process of evolution of one immunity protein into another. Specifically, we evolved Im9 (the cognate inhibitor of ColE9) towards inhibition of ColE7.

Results

Expression and activation of ColEs in emulsion compartments

Directed evolution of nuclease inhibitors is ideally performed *in vitro*, since all nucleases are toxic to living cells. We found that both the ColE7 and ColE9 genes translate efficiently *in vitro*, namely in cell-free extracts, and can be then activated by addition of divalent metals ions (Co^{2+} for ColE7, and Ni^{2+} for ColE9). The *in vitro* translated Im proteins were also active, since addition of cell-free extracts in which the Im7 or Im9 genes were translated, completely blocked the activity of the cognate ColE (Table 1). (For brevity, we refer to cell-free extracts in which a given gene was transcribed and translated, e.g. Im7, as Im7 cell-free extract.)

Nano-droplet solutions were prepared by adding aqueous solutions of bivalent salts (e.g. NiCl_2 ,

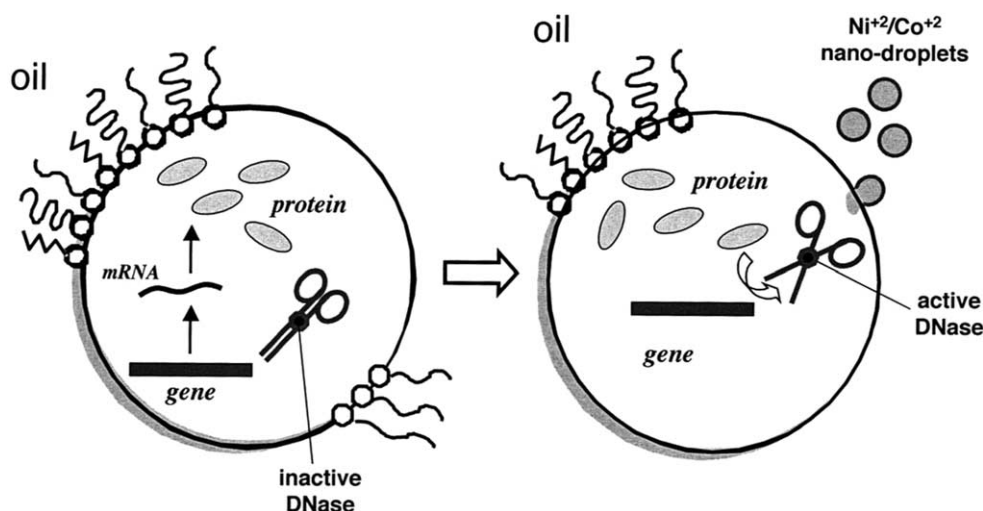


Figure 1. A schematic view of the selection system. A library of genes is added to a cell-free translation extract, and compartmentalized in the aqueous droplets of a water-in-oil (w/o) emulsion together with an inactive DNase. The genes are allowed to transcribe and translate, and the DNase is then activated through the delivery of nickel or cobalt ions by nano-droplets. Genes encoding a DNase inhibitor survive the digestion and are subsequently isolated and PCR amplified.

Table 1. Activity of DNase Cole9 in cell-free extracts

Sample	DNA survival (%)	
	Bulk assay ^a	Emulsion sample
Extract	100 ^b	100
Extract+ Cole9	59.4	25
Extract+ Cole9+ Ni ²⁺	≤5	1.5 ^c
Extract+ Cole9+ Im9+ Ni ²⁺	100	n.d.

n.d., not determined.

^a Assays in bulk solution were performed by incubation for 15 minutes of the DIG-biotin labeled DNA substrate with extracts expressing Cole9, with or without nickel ions, at 25 °C.

^b In emulsions composed of extract with no Cole9, the percentage of surviving DNA was essentially identical with or without the addition nickel ions.

^c DNA survival was as low as 0.01% when greater volumes of cell-free extracts expressing Cole9 were added (see Figure 4).

CoCl₂) to a 250-fold volume excess of mineral oil containing 7.5% (w/w) Span80 and 2.5%(w/w) Tween80. The mixture was shaken extensively until a clear solution was obtained. Analysis by light-scattering indicated the presence of droplets in the size of about 100 nm diameter (0.1 μm), indicating swollen micelles or nano-droplets with >30-fold smaller diameter than the emulsion droplets (Figure 2). The NiCl₂ nano-droplets solutions were then added to emulsions containing Cole9 cell-free extracts and 0.5 nM DNA substrate. The emulsions were incubated to allow DNA digestion to proceed, and then broken. The amount of undigested DNA substrate was determined by a nuclease activity assay and competitive PCR. In the absence of metal ions, DNA digestion was incomplete even after long incubations. However, a dramatic increase in the level of DNA digestion was observed following the addition of the nano-droplets nickel solution indicating that the nickel ions have indeed reached the aqueous droplets and activated the Cole9 (Table 1). DNA survival was even lower when larger volumes of Cole9 cell-free extracts were added (see Figures 3 and 4). The addition of the nano-droplets solutions had no significant effect on

the stability or size distribution of the emulsion droplets (data not shown).

Model selections of the DNase inhibitor Im9

Using the nano-droplets delivery system described above, genes encoding Im9 could be enriched from a large excess of ΔOPD genes encoding a protein with no inhibitory activity. The Im9 and ΔOPD genes were amplified from a construct carrying a T7 promoter and labeled with biotin at their 5' end. The Cole9 genes were translated in 10 μl of cell-free extract, and this extract (Cole9 cell-free extract) was added to fresh extract containing mixtures of the Im9 and ΔOPD genes in various ratios. The extract mixture was compartmentalized by emulsification to give, on average, ≤1 gene per compartment. The emulsions were incubated to complete the translation of the Im9 and ΔOPD genes within their respective compartments, and the nickel chloride nano-droplets were added to allow Cole9 activation and DNA digestion. The emulsions were broken, the DNA was captured from the aqueous phase onto streptavidin-coated magnetic beads and amplified by PCR. The results of these selections indicated ~100-fold enrichment for the genes encoding the inhibitor Im9 over the ΔOPD genes (Figure 3a). Starting from a ratio of 1:200 up to 1:2500 Im9/ΔOPD genes, the compartmentalized selections gave a mixture of these genes at ratios of ~1:3 down to about 1:20. No enrichment was observed without the addition of the nickel ion nano-droplets solution.

The recovery of Im9 genes surviving the compartmentalized selection process was estimated by competitive PCR against a third gene of a different length (Figure 3b). This experiment indicated that, under this selection pressure, ~0.3% of ΔOPD genes had survived, regardless of the initial concentration of the Im9 gene. The ratio of ΔOPD/Im9 gene after selection is ~3:1, and the fraction of Im9 genes that survived the selection is therefore ~0.1%. Since the initial fraction of Im9

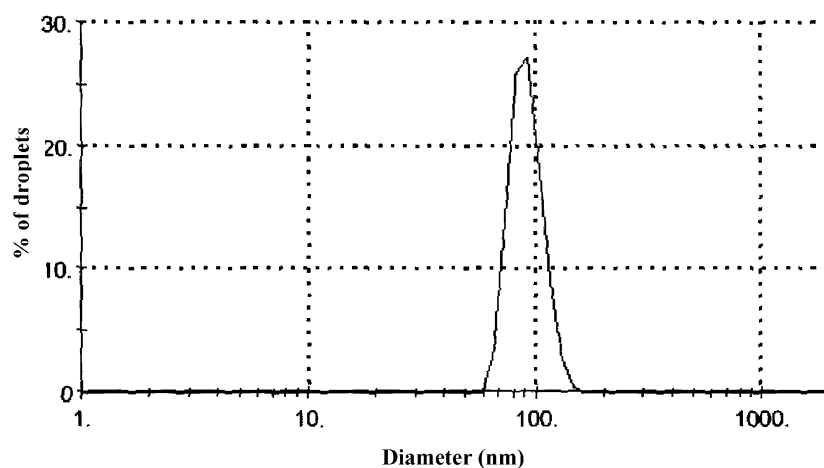


Figure 2. Size distribution of the nickel ion nano-droplets. The clear supernatant of a 250 mM NiCl₂ nano-droplets solution was analyzed by the light scattering HPPS instrument (Malvern Instruments). The size distribution analyzed either by number, and by volume (data not shown), gave a mean droplet diameter of ~100 nm.

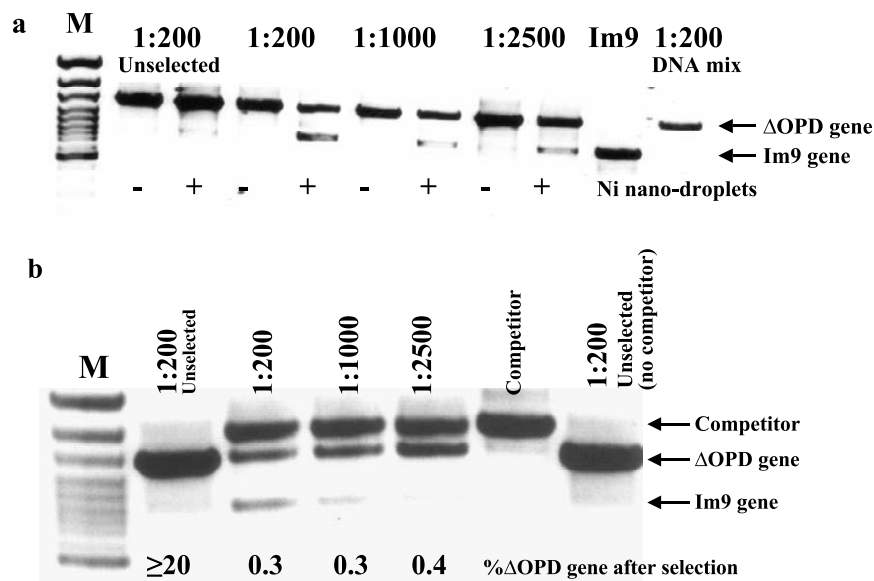


Figure 3. Model selections for the gene encoding the inhibitor Im9. The Cole9 cell-free extract was mixed with fresh extract containing mixtures of the Im9 and Δ OPD genes at various ratios (1:200, 1:1000, 1:2500 ratios of Im9/ Δ OPD). The extract mixture was then emulsified and incubated to allow the translation of genes in the compartments. Cole9 was activated by addition of NiCl₂ nano-droplets to half of the samples (labeled as Ni nano-droplets). The emulsions were broken, and the genes captured and amplified by PCR. a, Gel analysis of the PCR-amplified DNA (M, marker DNA (100 bp GeneRuler™, Fermentas); Unselected refers to a sample containing Im9 and Δ OPD biotinylated genes at a ratio of 1:200, emulsified without Cole9 extract; DNA mix refers to the original mixture of genes amplified with no prior treatment). b, The level of survival of the gene in excess (Δ OPD) was determined by competitive PCR. The PCR products were analyzed by agarose gel electrophoresis. The intensity ratio, between the Δ OPD and the competitor band, corresponds to the percentage of Δ OPD genes that survived the Cole9 digestion and is indicated in bold.

genes before selection was 1:200 (0.5%), the recovery of the Im9 genes is \sim 20%. Thus, the described selection procedure exhibits effective recovery of the “positive” genes (20%) and reasonable enrichments ($>$ 100-fold). Enrichment is limited primarily by a sizeable fraction of false positives (\sim 0.3%) due to genes that escape Cole9 digestion despite the absence of an inhibitor. Indeed, as shown below, much higher enrichments (\leq 500-fold enrichment, and 0.01% of undigested DNA; Figure 4) were obtained with this system when the efficiency of DNA digestion was improved by adding greater volumes of Cole9 cell-free extracts.

Selectivity and stringency of selection

For an evolutionary process to succeed, the selection pressure must change during its course. At the beginning, the selection pressure should be low to allow survival of all genes that encode a protein with the desired activity, be it low or high, so that no or little diversity is lost (high recovery). As the evolutionary process progresses, the selection pressure needs to be increased to allow genes encoding proteins with the highest activity to compete, thus leading to convergence rather than divergence of sequence (high enrichment). The selection system described here offers several ways in which the selectivity and stringency of the selection can be tuned.

An effective way of increasing selection pressure

is by changing the volume ratio between the Cole cell-free extract, and the fresh extract in which the immunity genes are translated. This increases the selection pressure in two ways: first, by increasing the concentration of the Cole nuclease; and second, by decreasing the translation levels of the immunity protein. In this way, the recovery of genes encoding an inhibitor with low affinity (e.g. a non-cognate immunity protein) can be easily tuned over a 50-fold range (from 0.7% down to 0.012%; Figure 4). Figure 4 also shows the selectivity of the selection since, in opposition to the low recovery of non-cognate immunity genes, \sim 10% of the cognate genes survive. The selection pressure can be further modulated by changing the incubation temperature and time with the nickel ion nano-droplets. The broad dynamic range of this selection system allowed us to control the threshold of the inhibitor’s affinity, and to perform library selections as described below.

Evolution of Im9 into a Cole7 inhibitor

We aimed at reproducing in the test-tube the evolution of a new specificity in an existing member of the immunity protein family. The diversification of natural immunity proteins is attributed mainly to high mutation rate during replication and to recombination.^{25,29,30} Random mutagenesis and homologous recombination were also used to diversify the Im9 gene for *in vitro* evolution, using

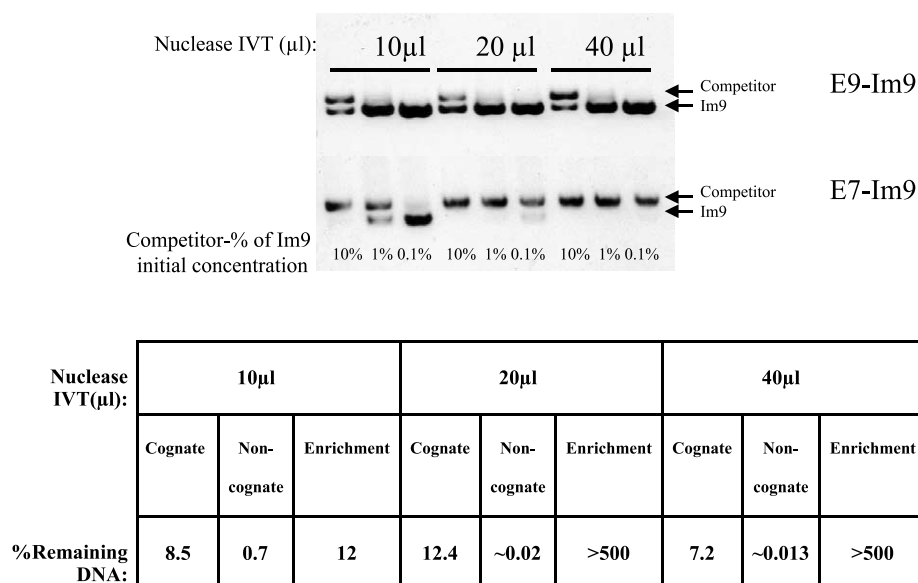


Figure 4. Selectivity and stringency of the selection pressure. Various volumes of cell free extracts (10–40 μl), in which either the Cole7, or Cole9, genes were translated at 4 nM, were mixed with aliquots of 100–70 μl of fresh extract containing 100 pM of the Im9 genes (total volume of 110 μl) and emulsified. The emulsion was incubated to allow the translation of Im9 gene and the colicin DNases were then activated by nano-droplets delivery of metal ions (24 hours at 25 °C followed by 30 minutes at 30 °C). The emulsions were broken, and biotinylated Im9 genes were captured on beads. The level of survival of the Im9 genes was determined by competitive PCR (see Materials and Methods). The competitor gene was added at amounts equivalent to 10%, 1% and 0.1% of the initial Im9 gene concentration. The products of the competitive PCR were analyzed on agarose gel and quantified by densitometry (Image Gauge v3.0). The ratio between the competitor and the Im9 gene provided an estimate of the survival of the selected Im9 gene. The results are summarized in the Table. The survival of the Im9 gene emulsified with a cognate DNase (Cole9) appears to be ~10%, regardless of the amount of Cole9 added. However, in the presence of the non-cognate Cole7, survival of the Im9 gene goes down, from 0.7% to 0.013%, as the volume of the Cole7 extract is increased. The enrichment corresponds to the ratio of survival of the Im9 gene in the presence of the cognate *versus* non-cognate colicin (Cole9 and Cole7, respectively).

error-prone PCR and DNA shuffling.³¹ Error-prone PCR in the presence of biased nucleotide ratios and manganese chloride was calibrated to an average mutation rate of two or three mutations per gene. This mutation rate gave the best enrichment and recovery. A library with higher mutation rate (13–20 mutations per gene) showed no enrichment after four rounds of selection. Additional mutations had accumulated during the numerous PCR cycles used

to amplify the surviving genes after each round of selection (an average of six mutations per gene was observed after rounds five and eight of the selection). The libraries of Im9 genes were selected for inhibition of Cole7. Following each round of selection, progress was monitored by competitive PCR to assess the percentage of surviving genes, and by assaying the inhibition activity of the pool of genes towards Cole7 (Figure 5).

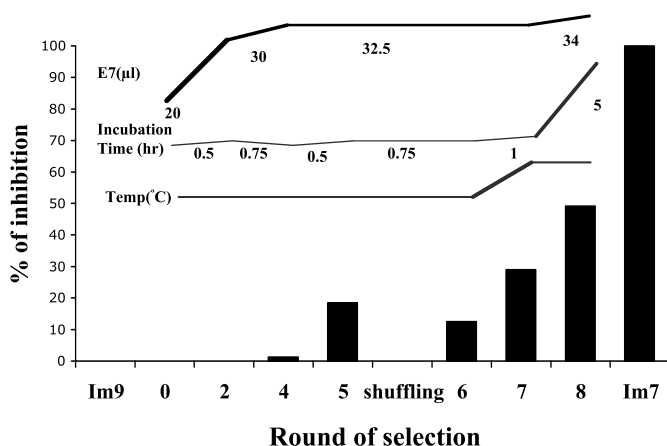


Figure 5. Progress of the selection of Im9 libraries for inhibition of Cole7. The surviving DNA from each round of selection (under conditions specified above the histogram), as well as wild-type Im7 and Im9 genes were translated and the resulting extract was mixed with activated Cole7. The percentage of undigested DIG-biotin DNA substrate was determined after ten minutes incubation at 30 °C. The survival in the presence of Im7 was defined as 100% inhibition.

The selection pressure was gradually increased, starting at a low selection pressure aimed at getting high recovery of genes (20 μ l ColeE7 cell-free extract, 200 pM selected DNA, and 0.5 hour incubation at 30 °C). As the evolutionary process progressed, we significantly increased the selection stringency (34 μ l of ColeE7 cell-free extract, 25 pM selected DNA, five hours incubation at 37 °C, in the last round of selection; Figure 5). By the fifth round, inhibitory activity of ColeE7 could be clearly observed. The pool of genes was cloned in *E. coli*, and sequencing of positive clones revealed several beneficial mutations at the “variable specificity region” of Im9, alongside mutations that seemed potentially damaging (e.g. a Ser to Pro, at position 65 in the middle of a helix). Backcrossing and homologous recombination of the selected clones were performed, by mixing the pool of genes from round 5 with wild-type Im9 at 1:1 ratio, and performing DNA shuffling.³¹ The shuffled library was subjected to three additional rounds of selection. The last round (round 8) was performed at high stringency (five hours incubation at 37 °C) as well as low stringency (one hour incubation at 37 °C).

The pool which survived the higher stringency conditions exhibited ~50% inhibition of ColeE7's DNase activity under conditions that yield 0% inhibition by Im9, and 100% by wild-type Im7 (Figure 5), whereas the pool of genes recovered from the less stringent selection condition (one hour incubation) showed about fourfold less activity. Both pools of genes were cloned in *E. coli*. Individual clones were amplified and the resulting DNA translated in cell-free extracts and assayed for inhibition of ColeE7 and E9. About half of the tested clones were found to effectively inhibit ColeE7 to various degrees (Table 2). As expected, several mutations in the variable specificity region, which appeared in separate clones from round 5 (e.g. Val 34Asp and Asp26Asn) were combined in single round 8 clones. In addition, several mutations that we suspected to be neutral or harmful disappeared: these include, Leu3Pro, Thr20Lys, Ser35Pro, Thr38Glu, Lys57Ser, Ser65Pro, Ser65Glu and Lys80Glu. The ability to modulate the stringency of selection was also manifested in the properties of individual immunity variants. Variants obtained from round 8 performed at low stringency, exhibited distinctly lower inhibitory activity (on average about threefold difference in activity; e.g. variant 1; Table 2) than those isolated from the high stringency selection (66–97%).

The newly evolved Im7 variants

The increased ability of the *in vitro* evolved variants to inhibit ColeE7 was confirmed by an *in vivo* protection assay.²⁷ Briefly, agar lawns of cells expressing the wild-type and newly evolved Im variants were grown and the plates were spotted with the ColeE7 toxin complex at various concentrations. Cell death was visualized in the form of a

plaque after overnight incubation, and the highest concentration of ColeE7 under which no cell death was apparent was recorded for each variant (Table 3). As previously observed,²⁷ these concentrations change with the level of Im protein expression as dictated by the level of isopropyl- β -D-thiogalactoside (IPTG) induction. The eighth round variants show a marked ability to protect against ColeE7 at concentrations that are 10^2 (no IPTG) up to 10^5 (1 mM IPTG) higher than Im9. The order of the *in vivo* protection capabilities roughly correlates with the order of inhibition seen with the *in vitro* assays (Table 2), with variant 1 being the poorest and 8 being the most potent.

Sequence analysis of round 8 clones (Table 2) showed convergence into residues at the variable specificity region of Im9, two of which (Asn26, Asp34) appear in wild-type Im7 (26 and 35 by Im7 numbering). These two residues are known to contribute significantly to binding of Im7 to ColeE7, *via* hydrogen and electrostatic bonds.^{32,33} The mutation Val34Asp seems to be the most significant source of improved ColeE7 inhibition, as indicated by the much lower inhibition exhibited by variant 1 that does not carry it. Other changes in the sequence of Im9 are characterized by the addition of negative charges (Asn24Asp, Lys57Glu), which is in agreement with Im7's specificity residues being of charged nature, compared to the more hydrophobic Im9.³⁴ In addition, conserved changes in residues 27 and 28 (Thr27Ala and Ser28Thr) were observed in most of the selected clones. The net effect of these substitutions, from polar into hydrophobic, is reasonable, since these residues are Ser and Thr in wild-type Im9, and Val and Ala in Im7. We presume that the mutations observed in residues 24, 27 and 28 have a smaller effect on activity as no significant change in inhibition was observed between variant that carry these mutations (e.g. variant 7) and variants that do not (e.g. variants 4 and 6). The high frequency of these mutations within the selected variants can be attributed to their linkage with other beneficial residues (these mutations seem to appear in clones of round 5, together with either Val34Asp or Asn24Asp), or simply due to the haphazard fixation of neutral mutations during the evolutionary process. Other mutations do not pose a dramatic change from wild-type residues (e.g. Val37Ile) yet their conservation suggests that they are of relevance. The rest of the mutations observed in the newly evolved Im variants are in areas that are remote from the colicin binding site region, and also vary from one variant to another (see Table 2, footnote b).

Only one selected variant appears to have a mutation in the conserved hot-spot in Tyr55 that confers a considerable degree of colicin binding energy in all immunity proteins (Tyr55Trp, variant 8). The activity assays (Table 2) and data by others on the same mutation,³⁵ suggest that this mutation does not lead to significant loss of binding affinity.

Table 2. Sequence and inhibition activity of the *in vitro* evolved immunity proteins

Position (Im9 numbering)	Immunity protein binding and selectivity-determining residues ^{a,b}																Inhibition of ColE7 (<i>in vitro</i> assay) ^c
	"Variable specificity region" and other residues												"Conserved hot-spot" residues				
	24	26	27	28	30	33	34	37	38	41	42	50	51	54	55	56	
Im9	Asn	Asp	Thr	Ser	Glu	Leu	Val	Val	Thr	Glu	Glu	Ser	Asp	Tyr	Tyr	Pro	0
Variant 1 ^d	Asp		Ala	Thr													33
Variant 7				Thr			Asp	Ile									69
Variant 4	Asp		Ala	Thr			Asp	Ile									72
Variant 6	Asp		Ala	Thr			Asp										66
Variant 8	Asp	Asn	Ala				Asp	Ile							Trp		97
Corresponding position in Im7	Lys	Asn	Val	Ala	Glu	Leu	Asp	Leu	Glu	Val	Lys	Thr	Asp	Tyr	Tyr	Pro	100

^a The Table lists all residues previously implicated in complex formation of both ColE9-Im9^{23,34} and ColE7-Im7,^{28,29} as well as Im9 residues in which highly conserved mutations were found in the newly evolved variants (e.g. residues 27 and 28).

^b Additional mutations observed in the newly evolved variants in residues that are, in most likelihood, not involved in colicin binding are: variant 7, Glu2Gly, Lys57Glu; variant A, Ser6Gly, Phe83Leu; variant 4, Lys57Glu; variant 6, Met43Thr; variant 8, Ser6Arg.

^c Inhibition of the DNase activity by the newly evolved variants, wild-type Im7 and Im9, was determined by bulk nuclease activity assay. The reaction mixtures were incubated at 33 °C in the presence of the DNA substrate for five minutes. Under these assay conditions, 100% inhibition was observed with cognate pairs and 0% with non-cognate.

^d Variants 1 and 7 were isolated from round 8 performed under low stringency conditions; all other variants were isolated from the high stringency selection (see the text).

Table 3. The inhibitory activity of the *in vitro* evolved immunity proteins in an *in vivo* protection assay

	w/o IPTG	0.05 mM IPTG	1 mM IPTG
Im7	$\geq 10^{-4}$	$\gg 10^{-4}$	$\gg 10^{-4}$
Clone 8	0.3×10^{-9}	0.3×10^{-7}	$> 10^{-4}$
Clone 4	10^{-9}	0.3×10^{-7}	$> 10^{-4}$
Clone 7	0.3×10^{-9}	1×10^{-8}	$> 10^{-4}$
Clone 6	0.3×10^{-10}	1×10^{-9}	0.3×10^{-8}
Clone 1	$< 10^{-11}$	10^{-11}	10^{-11}
Im9	10^{-11}	0.3×10^{-11}	0.3×10^{-10}
Δ O/PD	n.d.	n.d.	10^{-11}

Listed, for each Im variant, is the minimal ColE7 concentration (in M) at which full protection was observed.

Discussion

The newly developed *in vitro* evolution system

The nano-droplet delivery system presented here provides a general means of regulating biochemical processes that occur within the cell-like compartments and is of much utility. In this case, we could time the activation of a DNA-nuclease to enable gene translation to take place prior to DNA digestion. Previous works indicated few other ways of modulating the emulsion content without affecting its integrity. These include the delivery of hydrophobic substrates through the oil phase, reduction of pH by delivery of acetic acid, and photoactivation of a substrate contained within the aqueous droplets.¹⁹ The nano-droplet delivery significantly expands the scope of regulatory mechanisms. The high enrichment factors and recoveries indicate that the addition of nano-droplets of the type described above to water-in-oil emulsions has no undesirable effects on the integrity of the aqueous compartment or exchange of genes and proteins between droplets. The delivery of a variety of low-molecular mass, water-soluble ligands may also be helpful in regulating enzyme activities (by delivering allosteric effectors, for example) or gene expression (e.g. by IPTG-induced transcription of genes in cell-free extracts³⁶). Moreover, micelles as carriers into multiple emulsions have been reported for a variety of water soluble reagents³⁷ as well as enzymes.³⁸ Other compositions of nano-droplets or swollen micelles may also allow high-molecular mass molecules, e.g. DNA and proteins or even emulsion droplets,³⁹ to be delivered, as shown for entrapment of glucose oxidase.³⁸ The delivery of proteins or genes into emulsion droplets would be of much utility provided that it does not mediate the exchange of DNA or proteins between droplets and the subsequent loss of genotype-phenotype linkage.

Previous selections for nuclease inhibitors, including Im proteins, were performed using phage-display libraries and a selection for binding of the nuclease.^{27,40} In contrast, the nano-droplet delivery enabled us to establish a direct *in vitro*

selection for the inhibition of DNA nucleases. This selection system affords good enrichment factors (100–500-fold) and good recovery of inhibitor-encoding genes (~20%). The enrichment factor could be easily regulated in model selections of wild-type immunity genes (Figure 4), as well as in library selections for new immunity protein variants (Figure 5). In particular, adding greater volumes of ColE cell-free extracts does not only increase the number of ColE molecules per compartment, but also reduces the translation efficiency and hence the number of Im protein molecules. This results in a significant decrease in the Im/ColE ratio and thereby increases the stringency of selection and enrichment for high-affinity variants. This selection strategy may be applicable to other DNA-nucleases (be it endo or exo-nuclease) and perhaps to other DNA-modifying enzymes (DNA-methyltransferases, for example).

The *in vitro* evolution of new immunity protein variants

Cell-free translation allowed us to express three different ColE genes to yield enzymatically active nucleases. This provided a means of selecting immunity protein inhibitors in a completely *in vitro* fashion, and of circumventing the need to isolate the ColE protein after co-expression with their cognate immunity protein.⁴¹ ColEs were activated in cell-free extracts by addition of cobalt or nickel ions, but not by magnesium, as previously reported.^{23,24} It appears that these metals stabilize the structure of ColEs, a role that is suggested to be fulfilled also by immunity protein binding.²⁵

We selected new immunity protein variants out of a library derived from the Im9 gene. The unselected library exhibited almost no inhibition towards either ColE9 (the cognate nuclease of Im9) or ColE7 (the target of selection). The selection pressure was modulated through the rounds of selection to attain both high recovery and enrichment. By the fifth round of selection, individual variants were identified that showed some convergence towards specific sequence changes, which were then observed by the end of the selection process (round 8).

After eight rounds of selection, the inhibition activity of the best variants was still much lower than that of wild-type Im7, indicating that the evolutionary transition from Im9 activity into Im7 activity is clearly incomplete. Due to the need to express and purify colicins, and the very long dissociation half-lives of their complexes, the affinity of the newly evolved Im proteins is yet to be measured. Thus, to provide support for our *in vitro* assays, we followed the *in vivo* protection assays applied by Kleanthous and co-workers.²⁷ These assays correlate the affinity constants of Im protein variants with the degree of protection against ColE toxicity *in vivo*. The protection generally varies between K_d values that are $> 10^{-8}$ M (0% protection) and $K_d < 10^{-11}$ M (100% protection).

These protection assays show a dramatic increase in the ability of the selected Im9 variants to inhibit Cole7 (Table 3). Wild-type Im9, which binds Cole7 with a K_d value of 3.8×10^{-8} M, exhibited protection only at the lowest Cole7 concentrations (0.3×10^{-10} M, at the highest Im9 expression levels; Table 3). The best eighth round variants (4, 7 and 8) protect up to Cole7 concentrations of 10^{-4} M to 10^{-9} M, depending on the expression level of the Im proteins. The *in vivo* protection assays therefore suggest that these variants exhibit K_d values in the range of 10^{-10} to 10^{-11} M.

Although the Im variants were selected under conditions that are quite different from those prevailing in living *E. coli* cells, the selection pressure in the emulsion droplets led to an increased *in vivo* potency (Table 3). Another notable feature is the similarity in sequence changes between the newly evolved Im variants and their natural counterparts. All the meaningful sequence changes occurred at the variable specificity region around loop I and helix II, while the conserved hot-spot, at helix III (including Asp51, Tyr54 and Tyr55 of Im9) remained essentially unchanged (Table 2). In view of the completely random nature of the mutations in the unselected library, these results confirm the proposed mechanism of dual recognition, as well as the hypothesis regarding the routes by which colicin immunity interaction diverged during natural evolution. Thus, the conserved hot-spot appears to provide a common motif and a starting point for the evolution of new pairs, whereas divergence is mediated only by changes in the variable region (helix II) of the immunity protein.^{25,27} The role of the conserved hot-spot in providing an initial degree of cross-reactivity, and thereby a starting point for the evolution of new pairs is analogous to the possible role of enzyme promiscuity (or substrate ambiguity) in the evolution of new enzyme functions.⁴²⁻⁴⁴

Materials and Methods

DNA manipulation

The Cole9 and Im9, Cole2 and Cole7 genes were PCR-amplified from plasmids pKC67, pKH202 and pCole2, respectively (kindly provided by C. Kleantous) and cloned into pIVEX 2.2b (Roche) *via* NcoI and SacI sites to give pIVEX-E9, pIVEX-Im9, pIVEX-E2 and pIVEX-E7. Preparation of pIVEX- Δ OPD is described elsewhere.¹⁹ Im9 and Δ OPD PCR fragments for selection (Figure 1) were amplified using primers LMB2-2 Bc appending a biotin (biotin-5'-CAGGCTGCGCAACTGTTG-3') and LMB-3 (5'-GTCATAGCTGTTTCCTG-3'). The reactions were cycled 30 times (95 °C, 0.5 minute; 55 °C, 0.5 minute; 72 °C for 0.5–2 minutes depending on the fragments' length). The Cole2, Cole7 and Cole9 genes were PCR-amplified from the ligation mixtures of pIVEX-E9, pIVEX-Im9, pIVEX-E2 and pIVEX-E7, using primers LMB2-6 (5'-ATGTGCTGCAAGGCGATT-3') and pIVB-6 (5'-GTCGATAGTGGCTCCAA-3').

DNA from error-prone libraries, and the surviving DNA from each round of selection, were virtually cloned

into pIVEX, and amplified with biotinylated primers as described above. The DIG-biotin DNA substrate was amplified from a pIVEX vector carrying an insert which encodes the N-Flag and HA epitopes connected by a short linker, using primer LMB2-2 Bc appending a biotin, and LMB-3 appending a digoxigenin at the 5' end. The DNA fragments were all purified using the Wizard PCR Preps (Promega).

DNA digestion and nuclease activity assays

ColeE, Im, and Δ OPD genes were translated separately in Promega's S30 extract system for linear templates supplemented with T7 polymerase essentially as described.²¹ Unless otherwise specified, the DNA template concentration was 1 nM, and the reactions incubated for 2.5 hours at 25 °C. NiCl₂ or CoCl₂ were added to the translation extracts of Cole9 or Cole7, respectively, to a final concentration of 1 mM, followed by ten minutes incubation at room temperature or overnight at 4 °C. The translation extracts were then mixed at various nuclease/inhibitor ratios (1:1–1:4). The DIG-biotin DNA substrate was added to 5 nM concentration, and the digestion reactions incubated at 25 °C for various time periods. Aliquots at different time-points were quenched by 33-fold dilution in B&W buffer (1 M NaCl, 10 mM Tris, 25 mM EDTA, 15 mM EGTA (pH 7.4)). The 200 μ l of quenched solution was added to streptavidin-coated 96-well plates (Nunc) and incubated for one hour. The plates were rinsed three times with twice-concentrated B&W and PBS/T/BSA (PBS supplemented with 0.5% (v/v) Tween20 and 0.2% (w/v) BSA). A 200 μ l volume of a 1:1500 dilution in PBS/T/BSA anti-DIG-HRP conjugated antibody (Jackson) was added for one hour. The plates were rinsed three times with PBS/T and once with PBS, 200 μ l of TMB substrate (Dako) was added, and the $A_{405 \text{ nm}}$ measured.

Emulsions and nano-droplets delivery

The Cole9 gene was translated in cell-free extracts at 2 nM, for 2.5 hours at 25 °C. The DIG-biotin DNA substrate was added to 100 μ l of these extracts on ice, to a final concentration of 5 nM. The reaction mixture was added to 1 ml of ice-cold oil mix comprised of 4.5% (w/w) Span80, 0.5% (w/w) Tween80 in light mineral oil (Sigma), placed in a 2 ml cryotube (Corning). This emulsion mixture was kept in an ice-water bath and homogenized for five minutes at 8000 rpm in an IKA (Ultra Turrax T25) homogenizer equipped with a disposable shaft (OmniTip). The emulsions were then transferred to 25 °C. Nano-droplets systems were prepared by adding 250 mM NiCl₂ water solutions to 250-fold excess (v/v) of light mineral oil containing 7.5% (w/w) Span80 and 2.5% (w/w) Tween80. The mixture was extensively mixed (hard vortex followed by shaking), to obtain a clear solution. A precipitate would sometimes appear after longer incubations yet the clear supernatant was used in all cases to mediate the metal ion delivery. Then 500 μ l of NiCl₂ nano-droplet solution was added to the emulsion, followed by gentle mixing and 2–16 hours incubation at 25 °C.

To break the emulsion and isolate the genes, the emulsion was spun down at 10,600 g for five minutes. The oil phase was removed and 400 μ l of B&W buffer supplemented with 40 μ g/ml of yeast RNA, 25 mM EDTA and 15 mM EGTA, were added, followed by 1 ml of water-saturated ether. The tube was vortexed and the ether phase removed. The aqueous phase was rinsed

twice with ether, and traces of ether removed by SpeedVac drying for five minutes. The concentration of the DNA substrate in the samples was subsequently determined by nuclease activity assay as described above.

Model selections

The 100 μ l of ice-cold cell-free extracts containing 400 pM Δ OPD gene and various concentrations of the Im9 gene (2 pM, 0.4 pM or 0.16 pM; corresponding to 1:200, 1:1000 and 1:2500 ratios of Im to Δ OPD), were supplemented with 10 μ l of extract, in which the ColE9 gene was translated (3 nM template DNA, four hours at 25 °C). The extract mixture was emulsified as described above. The emulsion was incubated for four hours at 25 °C to allow the translation of the Δ OPD and Im9 genes. Then 500 μ l of NiCl₂ nano-droplet solution was added, and the mixture incubated for 16 hours at 25 °C. The emulsions were broken as described above, and the ether-rinsed aqueous phases were added to 200 μ l of B&W buffer plus 8 μ l of M280 streptavidin-coated magnetic beads (Dynal), and incubated for one hour. The beads were rinsed three times with twice-concentrated B&W buffer and three times with 5 mM Tris-HCl (pH 8), and then resuspended in 8 μ l of PCR buffer (16 mM (NH₄)₂SO₄, 67 mM Tris-HCl (pH 8.8), 0.1% Tween20). For PCR amplification, 2 μ l of bead suspension was diluted tenfold in PCR buffer corresponding to a 10⁵ dilution of the original DNA mix before selection, and amplified. Concomitantly, 0.4 pM of Im9 genes were similarly diluted and separately amplified. PCRs were performed with BioTaq (BioLine) for 30 cycles (95 °C, 0.5 minute; 63 °C, 0.5 minute; 72 °C, 1.5 minutes) using primers LMB2-6 and PIVB6. The PCR products were analyzed on 1% (w/v) agarose-TAE gels with DNA marker GeneRuler™ 100 bp ladder (Fermentas). Competitive PCR (Figures 3B and 4) was performed with the DNA solutions recovered from the emulsions described above. These were mixed with equal volumes of a competitor gene (an 1320 bp insert cloned into NcoI/SacI sites in pIVEX) at a concentration of 4 pM (corresponding to 1% of the initial concentration of Δ OPD gene used in selection). Then 1 μ l of this DNA mixture was diluted 100-fold in PCR buffer, and amplified in 20 μ l PCR reactions using primers LMB2-6 (Bc) and PIVB6 (Fo). The reactions were cycled 30 times, and the PCR products analyzed on 1% agarose-TAE gel.

Preparation of Im9 gene libraries

Randomization by error-prone PCR was based on described protocols.^{45,46} Briefly, 1 ng of pIVEX-Im9 DNA was amplified in PCR reactions containing NTPs (200 μ M in total) at 1:5 or 1:10 ratios of AC:TG, supplemented with 250 μ M MnCl₂, using the LMB2-9 and pIVB10 primers (25 cycles: 95 °C, 0.5 minute; 53 °C, 0.5 minute; 72 °C, 1.5 minutes in 1:5 bias, and two minutes in 1:10 bias). The PCR product was virtually cloned and amplified as described above. A fraction of the ligated pIVEX plasmid was transformed into DH5 α cells and several individual clones were sequenced to show a mutation rate of 1.14% and 1.64% in the 1:5 and 1:10 bias libraries. This percentage corresponds to an average of three and four mutations per gene (for the 1:5 and 1:10 bias libraries, respectively). Of the total mutations, 50% and 75%, bias 1:5 and 1:10, respectively, were transition mutations, and the rest transversion mutations, and 20% and 30% were synonymous mutations.

DNA shuffling was performed using existing

methods.³¹ Briefly, the pool of genes coming from the fifth round of selection was mixed with the wild-type Im9 gene at a 1:1 ratio. The DNA was digested with DNase I. DNA fragments of 75–125 bp length were gel-purified and PCR-assembled (100 ng DNA fragments: 94 °C, 0.5 minute, and then 35 cycles composed of a temperature gradient of 65–41 °C, 1.5 minutes at each temperature followed by 45 seconds at 72 °C). The PCR product was captured on M280 streptavidin-coated magnetic beads (Dynal). The beads were rinsed with twice-concentrated B&W buffer and PCR buffer. The bound DNA was PCR-amplified using primers LMB2-9, pIVB10 (18 cycles: 95 °C, 0.5 minute; 53 °C, 0.5 minute; 72 °C, one minute), digested by SphI and PstI (restriction sites upstream and downstream to NcoI and SacI sites, respectively), and virtually cloned into the pIVEX vector as described above.

Library selections

Selections were done essentially as the model selections described above. Each round was performed under changing DNA concentration, time and temperature of incubation (following the metal ion delivery by nano-droplets) as specified in Figure 5. After the first round of selection, the 1:5 and 1:10 bias libraries showed the same level of DNA survival and were combined into one library for the subsequent rounds of selection.

In vivo protection assays

These were performed essentially as described.²⁷ Briefly, the newly evolved, and wild-type Im variants were cloned into the IPTG-inducible expression plasmid pTrc99a (Pharmacia Biotech), and transformed to *E. coli* JM83 cells (kindly provided by C. Kleantous). Cells were grown as lawns on ampicillin-LB agar plates without, or with IPTG (0.05, or 1 mM), and spotted with ColE7 at different concentrations. Cell death was visualized in the form of plaques after overnight incubation, and the lowest concentration of ColE7 at which there was no formation of plaques was recorded (Table 3).

Acknowledgements

We are grateful to Colin Kleantous and Anthony Keeble for providing the ColE/Im plasmids, and their on-going guidance and support. Funding by an IMOS grant (Israeli Ministry of Science) and by the Dr Ernest Nathan Fund for Biomedical Research, is gratefully acknowledged. D.S.T. is the incumbent of the Elaine Bolnd Career Development Chair.

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Edited by J. Karn

(Received 3 August 2004; received in revised form 7 November 2004; accepted 9 November 2004)