Biotechnology
Paper alert

A selection of interesting papers that were published in the two months before our press date in major journals most likely to report significant results in biotechnology.

- • of special interest
- •• of outstanding interest

Current Opinion in Biotechnology 2002, 13:523–530

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Analytical biotechnology

Selected by Catherine Halliwell and Tony Cass
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•• Oligosaccharide microarrays for high-throughput detection and specificity assignments of carbohydrate–protein interactions. Fukui S, Feizi T, Galustian C, Lawson AM, Chai W: *Nat Biotechnol* 2002, 20:1011-1017.
Significance: Carbohydrate–protein interactions mediate a large number of both normal and pathophysiological processes in humans. Insight into the nature and specificity of these interactions is complicated by the complex structures of glycoconjugates. High-throughput analysis based on a microarray format offers a route to efficient mapping of these interactions.
Findings: A variety of oligosaccharides, both synthetic and tissue-derived were arrayed on nitrocellulose membranes with retention of their biological activity. Subsequently, the specificity of protein binding could be probed with enzyme-labelled antibodies and selectins (carbohydrate-binding proteins). Arrays in both mini (4 mm spot) and micro (300 μm spot) formats were generated and further deconvolution of binding specificity was achieved by *in situ* mass spectrometry.

• A protease assay for two-photon cross-correlation and FRET analysis based solely on fluorescent proteins. Kohl T, Heinze KG, Kuhlemann R, Koltermann A, Schwille P: *Proc Nat Acad Sci USA* 2002, 99:12161-12166.
Significance: There is increasing interest in measuring enzyme activities *in situ* in living cells. Reagents to achieve this can be introduced from the outside or expressed as recombinant proteins. Of the latter, naturally occurring fluorescent proteins have the advantage of being amenable to fluorescence measurements with a sensitivity and duration that is compatible with live-cell imaging.
Findings: Fusion proteins of two naturally occurring fluorescent molecules (green fluorescent protein [GFP] and Discosoma red fluorescent protein [DsRed]) via a protease-sensitive linker sequence were constructed and the fluorescence of the two proteins (measured by two-photon excitation and dual-colour fluorescence correlation spectroscopy determined. Although the two proteins show a fluorescence resonance energy transfer (FRET) that is abolished by cleavage of the linker, the nature of FRET means that the two fluorophores are constrained in the distance by which they are separated. In contrast the cross-correlation, which measures fluctuations in emission intensity as a result of diffusion, is applicable to any separation of the two fluorophores. A proof-of-principle is provided by measuring the activity of the tobacco etch virus (TEV) protease using a GFP–DsRed fusion protein with a linker carrying the protease-recognition sequence.

• Dual enzyme electrochemical coding for detecting DNA hybridization. Wang J, Kawde A-N, Musameh M, Rivas G: *Analyst* 2002, 127:1279-1282.
Significance: Multiplex detection of DNA hybridisation most commonly employs optical detection using labels such as light-scattering particles, quantum dots or organic dyes. Various coding schemes are used to distinguish the different signals and these may be physical, spectroscopic or spatial. Although much less widely used, electrochemical detection methods may be employed and can exploit the different oxidation potentials of electroactive products of enzyme activity.
Findings: Two DNA probes were employed, each labelled with a different enzyme (β-galactosidase and alkaline phosphatase). The electroinactive substrates for the two enzymes are converted to electroactive products with different oxidation potentials. The use of magnetic bead separation methods and chronopotentiometric measurements demonstrated that this approach could be used to detect two different DNA sequences.

Plant biotechnology

Selected by Jim Dunwell
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•• Regulation of transgene expression in plants with polydactyl zinc finger transcription factors. Ordiz MI, Barbas CF III, Beachy RN: *Proc Natl Acad Sci USA* 2002, 99:13290-13295.
Significance: Designer zinc-finger transcription factors (TFs(ZF)) have been developed to control the expression of transgenes and endogenous genes in mammalian cells. Application of this technology in plants, as described in this study, would enable a wide range of both basic and applied studies.
Findings: The authors report the use of TFs(ZF) to target a defined 18 base pair DNA sequence to control gene expression in plant cells and in transgenic plants. A β-glucuronidase reporter gene was activated using the designed six-zinc-finger

protein 2C7 expressed as a fusion with the herpes simplex virus VP16 transcription factor activation domain. Reporter gene expression was activated 5- to 30-fold by using TFs(ZF) in BY-2 protoplasts, whereas expression was increased as much as 450 times in transgenic tobacco plants. Such plants that produce 2C7 transcription factors were phenotypically normal through two generations, suggesting that the factors exerted no adverse effects.

•• A light-switchable gene promoter system. Shimizu-Sato S, Huq E, Tepperman JM, Quail PH: *Nat Biotechnol* 2002, 20:1041-1044.
Significance: Regulatable transgene systems providing easily controlled, conditional induction or repression of expression are indispensable tools in biomedical and agricultural research and biotechnology. Most systems developed for eukaryotes rely on the administration of either exogenous chemicals or heat shock and, despite their general success, there are potential problems, such as toxic, unintended or pleiotropic effects of the inducing chemical or treatment. The system reported here involves the development of a promoter system that can be induced, rapidly and reversibly, by short pulses of light, and may be of particular value in controlling gene expression in plants.
Findings: This system is based on the known red-light-induced binding of the plant photoreceptor phytochrome to the protein PIF3 and the reversal of this binding by far-red light. The authors show that yeast cells expressing two chimeric proteins, a phytochrome–GAL4-DNA-binding domain fusion and a PIF3–GAL4-activation-domain fusion, are induced by red light to express selectable or 'scorable' marker genes containing promoters with a GAL4 DNA-binding site, and that this induction is rapidly abrogated by subsequent far-red light. They further showed that the extent of induction could be controlled precisely by titration of the number of photons delivered to the cells by the light pulse. Thus, this system has the potential to provide rapid, noninvasive, switchable control of the expression of a desired gene to a preselected level in any suitable cell by simple exposure to a light signal.

• Production of pineapple transgenic plants assisted by temporary immersion bioreactors. Espinosa P, Lorenzo JC, Iglesias A, Yabor L, Menendez E, Borroto J, Hernandez L, Arencibia AD: *Plant Cell Reports* 2002, 21:136-140.
Significance: Despite the general success in developing transformation technologies for a wide range of crops, further improvements are desirable, particularly for tropical species many of which have considerable economic value. The novel procedure reported here for producing transgenic pineapple (*Ananas comosus* (L.) Merr.) plants using temporary immersion bioreactors (TIBs) may be more generally applicable.
Findings: Initial experiments showed that pineapple calluses ranging in size from 1.5–2.0 mm that were co-cultivated with *Agrobacterium tumefaciens* strains AT2260 (pIG121Hm) and LBA4404 (pTOK233) for 24 h produced the highest percentage (40%) of glucuronidase-positive calluses. Phosphinothricin and hygromycin, but not kanamycin, were effective subsequently as selective agents for material growing in TIBs. Large-scale transformation experiments resulted in up to a 6.6% efficiency of transgenic plant production. TIB technology was found to be more efficient for transgenic plant selection than conventional micropropagation. Polymerase chain reaction and genomic Southern blot analyses confirmed the nonchimeric nature of the transgenic plants recovered from TIBs.

Environmental biotechnology

Selected by Lawrence P Wackett
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•• Hydroxyectoine is superior to trehalose for anhydrobiotic engineering of ***Pseudomonas putida*** KT2440. Manzanera M, Garcia de Castro A, Tondervik A, Rayner-Brandes M, Strom AR, Tunnacliffe A: *Appl Environ Microbiol* 2002, 68:4328-4333.
Significance: Desiccation tolerance is an important bacterial phenotype in nature and in the biotechnological application of microbes for bioremediation or biopesticides. Making sensitive bacteria desiccation tolerant requires adding genes for the biosynthesis of osmoprotectants or adding them to their surroundings. This study looked at both strategies for an organism that has potential environmental applications.
Findings: *Pseudomonas putida* KT2440 was genetically engineered to produce higher intracellular levels of the osmoprotectant trehalose. The resultant recombinant strain did not show increased desiccation tolerance; however, addition of hydroxyectoine to cultures resulted in significantly enhanced desiccation tolerance. Forty to sixty percent viability was maintained over extended periods in the presence of hydroxyectoine.

•• Harnessing microbially generated power on the seafloor. Tender LM, Reimers CE, Stecher HA, Holmes DE, Bond DR, Lowy DA, Pilobello K, Fertig SJ, Lovley DR: *Nat Biotechnol* 2002, 20:821-825.
Significance: *In situ* human activities in remote deep sea regions require a source of power to drive equipment. Microbes create electrical gradients in these environments. Harvesting this potential energy source could be useful, for example, in powering a series of deep sea sensors that would then not require batteries or maintainance.
Findings: Fuel cells consisting of an anode embedded in sediments and a cathode in overlying seawater were placed in two natural sea environments. Both cells were maintained for months and proved to be quite stable, producing a constant power output of greater than 0.025 W/m^2. Some of the bacterial composition in the sediment fraction of the power cell was investigated. delta-Proteobacteria, belonging to the family Geobacteraceae, comprised the majority of the bacteria present.

•• Polyphosphate kinase from activated-sludge performing enhanced biological phosphorus removal. McMahon KD, Dojka MA, Pace NR, Jenkins D, Keasling JD: *Appl Environ Microbiol* 68:4971-4978.
Significance: Bacterial polyphosphate formation in activated-sludge is the key metabolic component of enhanced biological phosphorus removal. High phosphorus in water causes algal blooms and other undesirable ecological effects, it is therefore important to better understand the microbiology underlying enhanced biological phosphorus removal. This has been difficult, however, because many of the complex microflora, including some implicated in phosphorus removal, have been difficult to cultivate in the laboratory. Molecular methods are currently used to identify components of the complex microbial ecosystem.
Findings: Polymerase chain reaction (PCR) and fluorescent *in situ* hybridization methods were used to identify the dominant organism in activated-sludge to be one resembling *Rhodocyclus*. PCR was further used to obtain putative genes encoding polyphosphate kinase from organisms in the sludge.

One of the genes was cloned, sequenced and the type I polyphosphate kinase purified to near homogeneity.

•• Novel cyanobacterial biosensor for detection of herbicides. Shao CY, Howe CJ, Porter AJ, Glover LA: *Appl Environ Microbiol* 2002, 68:5026-5033.
Significance: Biological sensors for the detection of herbicides in the environment could provide cheaper and faster methods for monitoring soils and waters. Cyanobacteria can be sensitive indicator organisms, because herbicides often inhibit photosystems or cause other metabolic perturbations that can be measured. The present study used a cloned luciferase gene from the firefly *Photinus pyralis* and light emission as an indicator of cell metabolic health, and thus herbicide presence and concentration.
Findings: The light emission system showed predictable behavior over a wide pH range. In water, seven different herbicides could be detected: diuron, paraquat, mecorpop, glyphosate, atrazine, simazine and propazine. The toxic effects on light output were measured for a number of the herbicides. Heavy metals and a volatile organic compounds also showed pronounced effects on light emission.

•• Cloning, sequencing, and characterization of the hexahydro-1,3,5-trinitro-1,3,5-triazine degradation gene cluster from ***Rhodococcus rhodochrous***. Seth-Smith HM, Rosser SJ, Basran A, Travis ER, Dabbs ER, Nicklin S, Bruce NC: *Appl Environ Microbiol* 2002, 68:4764-4771.
Significance: Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a widely used explosive that has become an environmental contaminant near places of its manufacture and disposal. Biotechnological approaches to RDX remediation, however, have been hampered by a lack of information on the underlying molecular basis of its degradation. This study begins to fill that knowledge gap.
Findings: The bacterium *Rhodococcus rhodochrous* was isolated for its ability to metabolize RDX as a nitrogen source. Products from RDX were determined to be nitrate, formaldehyde and formate. A gene region involved in RDX metabolism was identified and found to encode a cytochrome P450-like protein with a flavodoxin domain and an adrenodoxin reductase homolog.

Protein technologies and commercial enzymes

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• Structure-based combinatorial protein engineering (SCOPE). O'Maille PE, Bakhtina M, Tsai MD: *J Mol Biol* 2002, 321:677-691.
Significance: This paper proposes a new protein engineering approach based on structural information combined with existing directed evolution methods.
Findings: In this work the authors take two distantly related DNA polymerase enzymes, Pol X and Pol β, that share low sequence homology but have some similar secondary structure and create a cross-over library from the two genes, based on equivalent secondary structure elements. The resulting library was tested using complementation in *Escherichia coli* and several novel polymerases were found. It is seen that both the secondary structure elements and the linkages between them are important in determining activity. This approach could be useful for completing the range of applications for directed evolution techniques, as it is homology independent but does not rely simply on enzymatic digestion of the gene.

• Stable self-assembly of a protein engineering scaffold on gold surfaces. Terrettaz S, Ulrich WP, Vogel H, Hong Q, Dover LG, Lakey JH: *Protein Sci* 2002, 11:1917-1925.
Significance: A mutant of the *E. coli* protein OmpF has been created, which binds directly to gold surfaces and provides an anchor to link other proteins by further engineering.
Findings: The pore-forming outer-membrane protein OmpF has been engineered to include a single cysteine residue on the periplasmic surface. The resulting mutant is deposited onto a gold surface that had been previously treated with β-mercaptoethanol. Once the protein is immobilised thiolipids are added, forming a proteolipid layer at the gold surface. The conformation of OmpF was tested using binding of the R-domain of toxin colicin N, which is monitored by surface plasmon resonance and ion channel blockage. The gold surface denatures some of the otherwise highly robust OmpF, but functional protein is still present at high density. Although this technique could be applied to other outer-membrane proteins the interest here from a protein engineering standpoint lies in the possibility of creating OmpF fusions that can be easily purified and assembled onto the gold surface with relative ease. This technology could have applications in the field of biosensor construction.

• A positive charge preservation at position 116 of αA-crystallin is critical for its structural and functional integrity. Bera S, Thampi P, Cho WJ, Abraham E: *Biochemistry* 2002, 41:12421-12426.
Significance: Reports that autosomal dominant congenital cataracts are associated with a missense mutation (Arg116Cys) in the sequence of human αA-crystallin are further examined in this study. The study demonstrated that preservation of a positive charge at position 116 of the protein is critical in preserving the structure and function of the protein.
Findings: Arg116 in human αA-crystallin was mutated to lysine (Arg116Lys), cysteine (Arg116Cys), glycine (Arg116Gly) and aspartic acid (Arg116Asp). Both wild-type and mutant proteins were expressed, purified and characterised by measurements of circular dichroism, tryptophan fluorescence, 2-(*p*-tolidino) naphthalene-6-sulfonic acid (TNS) fluorescence and chaperone function. The mutant Arg116Lys had similar structure and function to the wild type. However, mutation of the residue to an acidic residue (Arg116Asp) showed drastic changes in protein structure. Mutation of the arginine to cysteine or glycine showed similar changes in structure, oligomerization and chaperone function, thereby suggesting that the presence of the cysteine does not cause the changes. Therefore, a positive charge at residue 116 is essential for preserving the structure and function of αA-crystallin.

• Arginine 165/Arginine 277 pair in (***S***)-mandelate dehydrogenase from ***Pseudomonas putida***: role in catalysis and substrate binding. Xu Y, Dewanti AR, Mitra B: *Biochemistry* 2002, 41:12313-12319.
Significance: Using site-directed mutagenesis the role of the conserved residue Arg165 in (*S*)-mandelate dehydrogenase and the overall importance of the Arg165/Arg277 pair were examined. It was found that although Arg165 plays a role in substrate binding and catalysis, its role is not as important as that of Arg277.

Findings: Single-mutants at Arg165 as well as double-mutants at Arg165 and Arg277 were characterised kinetically and functionally. The results showed that Arg165 stabilizes the transition state through its positive charge and stabilizes the ground state through a charge-independent interaction. The k_{cat} for the Arg165Lys/Arg277Lys mutant was found to be ~350-fold lower than wild type. This demonstrated that at least one arginine residue was required for optimal substrate orientation and catalysis. Studies of pH showed that a pK_a of 9.3 in free wild-type enzyme did not belong to Arg165 and suggested that it belonged to Arg277. Therefore, Arg277 is the more critical residue in terms of catalysis and substrate binding in (*S*)-mandelate dehydrogenase.

• Rational cytokine design for increased lifetime and enhanced potency using pH-activated 'histidine switching'. Sarkar CA, Lowenhaupt K, Horan T, Boone TC, Tidor B, Lauffenburger D: *Nat Biotechnol* 2002, 20:908-913.
Significance: The authors provide a rational strategy through which more potent analogs of specific protein therapeutics might be designed.
Findings: Drugs typically rely on the ability of ligands to bind tightly to their target. However, the sorting of complexes involving growth factors or cytokines is related to the endosomal ligand-receptor binding affinity, with complexes that remain bound to the receptor generally becoming degraded and those that dissociate being recycled. Redesigning a cytokine to decrease endosomal affinity after internalisation could reduce receptor downregulation and ligand depletion, enhancing drug effectiveness. This rationale takes advantage of the pH decrease from 7 to 5–6 in the endosomes, and is based on the principle that neutral histidine could maintain relatively tight binding on the cell surface, but protonated histidine could lead to weaker binding in the endosomal compartment. Candidate mutation sites were identified in areas of excessive negative charge density and in areas with electrostatic complementarity to the granulocyte colony-stimulating factor (GCSF) and its receptor. Calculation of the electrostatic contribution for wild type and each of the histidine mutants shows that for three of them (Glu20His, Asp110His, and Asp113His) the charged form has about 5 kcal/mol higher binding free energy than the neutral form. The K_d determined for the selected candidates (Asp110His and Asp113His) show that while binding is unaffected at pH 7.4, it weakens at pH 5.5 (K_d up to sevenfold higher at pH 5.5 for Asp113His). Tests on ligand depletion demonstrate that each of the mutants shows a much greater half-life (>500 h) than the wild type (50 h). Finally, an enhancement of ligand recycling was found in the case of the two mutants, validating the goal set by the authors.

• Conversion of a transmembrane to a water-soluble protein complex by a single point mutation. Tsitrin Y, Morton CJ, El Bez C, Paumard P, Velluz MC, Adrian M, Dubochet J, Parker MW, Lanzavecchia S, van Der Goot FG: *Nat Struct Biol* 2002, 9:729-733.
Significance: This paper presents the conversion of the pore-forming toxin aerolysin into a soluble complex by simple point mutation.
Findings: Pore-forming aerolysin is a four-domain protein that undergoes oligomerisation, following C-terminal proteolytic cleavage, to form a ring-like heptameric structure with amphipathic properties that allow it to insert into the lipid membrane. The upper boundary of domain 4 is delineated by a row of three aromatic residues (Tyr298, Phe410, and Tyr221), which are reminiscent of the 'aromatic belt' found in other membrane proteins, and are thought to anchor and stabilise the protein in the bilayer. The authors analysed the importance of these aromatic residues by mutating each to a glycine, and showed that the Tyr221Gly mutation completely blocked hemolysis. The circular dichroism (CD) spectra of Tyr221Gly showed no difference from the wild type. By contrast, the near-UV CD spectrum of Tyr221Gly heptamers differed from that of the wild type, indicating that their tertiary structure must differ despite the similarity of their secondary structures. Cryo-negative staining electron microscopy (EM) showed that the wild-type heptamers formed irregular, mostly aggregated structures and micelle-like structures. In contrast, regular structures were observed for the Tyr221Gly mutant, suggesting that these heptamers do not expose hydrophobic surface, as further confirmed by 8-anilo-1-naphthalene-6-sulfonic acid (ANS) binding experiments. The 13.5 Å model generated from the EM data provide some clues about the activation of the toxin into a membrane-bound state.

• Rational design of green fluorescent protein mutants as biosensor for bacterial endotoxin. Goh YH, Frecer V, Ho B, Ding JL: *Protein Eng* 2002, 15:493-502.
Significance: Virtual mutagenesis was used to design mutants of enhanced green fluorescent protein (eGFP) with high affinities for bacterial endotoxin, lipopolysaccharide (LPS), and its bioactive component, lipid A (LA). The most successful rationally designed eGFP mutant, G10, captured LA with a dissociation constant of 8.5 μm and exhibited the highest attenuation of fluorescence intensity in the presence of LPS/LA.
Findings: Previous work by this group showed that LPS or LA can interact and bind to short cationic sequences containing symmetrical amphipathic β-sheet motifs composed of alternating basic, hydrophobic and polar residues, with dissociation constants in the micromolar range. Virtual mutagenesis of an eGFP model suggested the most likely position of this motif for successful LPS/LA detection. Several mutants of eGFP (G10–G12) were then made and mutant G10, which has amino acids 200-YLSTQ-204 mutated to KLKTK, was found to have the highest level of fluorescence quenching in the presence of LPS/LA. Further development of the G10 mutant may provide the basis for a fluorescent biosensor for bacterial endotoxins.

• Excretion of human β-endorphin into culture medium by using outer membrane protein F as a fusion partner in recombinant ***Escherichia coli***. Jeong KJ, Lee SY: *Appl Environ Microbiol* 2002, 68:4979-4985.
Significance: A novel OmpF fusion system was developed for the production of recombinant proteins in *Escherichia coli* BL21 (DE3) to be excreted into the culture medium in large amounts. To show the validity of the system, human β-endorphin was used as a model protein. Using the OmpF fusion system 545 mg of β-endorphin protein were obtained from 2.7 L of culture supernatant.
Findings: A variety of methods have been developed for the excretion of recombinant proteins from *E. coli*, unfortunately most give poor yields of the target protein. This group have developed a system using a combination of an OmpF fusion protein and an OmpF knockout strain of *E. coli* BL21(DE3). Human β-endorphin was used as a test case for the system with good results. The protein was excreted into the culture medium, from which it was successfully purified. OmpF was then cleaved from β-endorphin using an engineered Factor Xa cleavage site. This novel system provides high yields of a

biologically important protein and should be applicable to the production of other recombinant proteins.

Expression vectors and delivery systems

Selected by Thomas A Kost and Patrick Condreay
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•• A light-switchable gene promoter system. Shimizu-Sato S, Hug E, Tepperman JM, Quail PH: *Nat Biotechnol* 2002, 20:1041-1044.
Significance: This is the first description of the development of a light-based inducible gene promoter system for regulating gene expression in eukaryotes.
Findings: A light-inducible promoter system was developed based on the red-light-induced binding of the plant photoreceptor phytochrome to the protein PIF3 and the reversal of binding by far-red light. Yeast cells expressing two chimeric proteins, a phytochrome–GAL4-DNA-binding domain fusion and a PIF3–GAL4-activation domain fusion, were induced by red light to express reporter genes regulated by a promoter containing a GAL-4 binding site. The induction could be rapidly reversed by subsequent exposure to far-red light. In this system background expression was almost undetectable and the reporter gene product was produced within 5 min of a 1 min pulse of light. The system does have limitations. Cells cannot be exposed to ambient light, as the red component light would result in induction of the promoter. Also, the cells must contain the phycocyanobilin cofactor. Yeast readily takes up this molecule, but the authors did not comment on the ability of mammalian cells to take up this compound. It will be of great interest to see if this system works as well in mammalian cells as it does in yeast.

• Transposition from a gutless adeno-transposon vector stabilizes transgene expression ***in vivo***. Yant SR, Ehrhardt A, Mikkelsen JG, Meuse L, Pham T Kay MA: *Nat Biotechnol* 2002, 20:999-1005.
Significance: The development of a gene-deleted adenovirus vector containing the *Sleeping Beauty* transposase provides a novel system for the stable maintenance of adenovirus-delivered transgenes.
Findings: A gene-deleted adenovirus vector containing the *Sleeping Beauty* transposase undergoes Flp-mediated recombination and excision of its expression cassette in the presence of flp recombinase. Systemic *in vivo* delivery of this system resulted in the efficient generation of transposon circles and stable transposase-mediated integration of the transgene in mouse liver hepatocytes. Somatic integration resulted in the maintenance of therapeutic levels of human coagulation Factor IX for more than six months in mice undergoing liver regeneration. These vectors provide a useful approach for adenovirus-mediated stable gene delivery studies *in vivo*.

• Engineered viruses to select genes encoding secreted and membrane-bound proteins in mammalian cells. Moffatt P, Saloia P, Gaumond M-H, St-Amant N, Godin E, Lanctot C: *Nucleic Acids Res* 2002, 30:4285-4294.
Significance: This virus-based signal sequence trap system allows for the rapid isolation and identification of cDNAs encoding secreted and membrane-bound proteins.
Findings: A sindbis virus replicon was engineered such that the envelope protein precursor no longer enters the secretory pathway. cDNA fragments were fused to the mutant precursor and expression screened for their ability to restore membrane-localization of the viral envelope proteins. In this manner, recombinant replicons were released into the culture medium only if the cDNA fragment they contained encoded a secretory signal. Of 2564 inserts that were retrieved after screening various cDNA libraries, more than 97% were shown to contain a putative signal peptide. This system appears to be highly sensitive and efficient for detecting and isolating signal-sequence-containing cDNAs.

• A monomeric red fluorescent protein. Campbell RE, Tour O, Palmer AE, Steinbach PA, Baird GS, Zacharias DA, Tsien RY: *Proc Natl Acad Sci USA* 2002, 99:7877-7882.
• A far-red fluorescent protein with fast maturation and reduced oligomerization tendency from ***Entacmaea quadricolor*** (Anthozoa, Actinaria). Wiedenmann J, Schenk A, Röcker C, Girod A, Spindler K-D, Nienhaus GU: *Proc Natl Acad Sci USA* 2002, 99:11646-11651.
Significance: Availability of a red fluorescent protein (RFP) that does not form oligomers will allow the formation of genetic fusions to proteins to visualize the cellular localization of the fusion partner.
Findings: The commercially available RFP from *Discosoma* species (DsRed) normally forms tetramers. Mutagenesis of residues found on oligomerization surfaces led to variants that form dimers and eventually a monomeric form, although these variants had disabled fluorescence. Further mutagenesis led to a monomeric RFP (mRFP1) that contains 33 mutations that restore the protein's red fluorescence. Fusion of mRFP1 to connexin43 led to the formation of functional, red-fluorescing gap junctions. The second manuscript describes the characterization of an RFP from *Entacmaea quadricolor* (eqFP611). This protein exhibits favorable emission wavelength and maturation kinetics, and may be monomeric in low concentrations. One limitation is the ability to express the protein in functional form only at low temperatures.

• Measuring the pH environment of DNA delivered using nonviral vectors: implications for lysosomal trafficking. Akinc A, Langer R: *Biotechnol Bioeng* 2002 78: 503-508.
Significance: A method is described that can help predict the ability of non-viral transfection reagents to bypass lysosomal degradation and thus be more effective gene delivery tools.
Findings: A method to measure the intracellular pH environment of formulated DNA samples is described. This method allows a prediction to be made as to whether or not a DNA sample that is delivered by formulation with various transfection reagents is able to bypass trafficking to acidic lysosomes where the DNA is most likely degraded. In general, a correlation was seen between the efficiency of gene delivery mediated by a given reagent and the neutrality of the intracellular pH environment of the DNA.

• Construction and characterization of thermo-inducible vectors derived from heat-sensitive ***lacI*** genes in combination with the T7 A1 promoter. Chao Y-P, Chern J-T, Wen C-S, Fu H: *Biotechnol Bioeng* 2002, 79:1-8.
Significance: Improvements in methods of induction of recombinant protein production in bacteria are important for the large-scale use of these systems.

Findings: A system employing the *lacI* gene to control expression of *lacZ* protein from a T7 A1 promoter containing two *lac* operator sites was used to introduce mutations into the *lacI* gene to confer thermo-inducibility on the repressor. The ability of these mutant repressors to repress expression at low temperature, yet allow high-level expression upon temperature induction, was investigated. Mutations at positions 241 or 265 of the repressor protein demonstrate 400- and 220-fold induction of expression, respectively, after temperature shift. Furthermore, this system appears to be adaptable to fermentor systems.

Pharmaceutical biotechnology

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•• N-Alkyl urea hydroxamic acids as a new class of peptide deformylase inhibitors with antibacterial activity. Hackbarth CJ, Chen DZ, Lewis JG, Clark K, Mangold JB, Cramer JA, Margolis PS, Wang W, Koehn J, Wu C, Lopez S *et al.*: *Antimicrob Agent Chemother* 2002, 46:2752-2764.

Significance: In the hunt for novel antibacterial agents, especially those with activity against resistant pathogens, target-based drug discovery has been famously unsuccessful. This study demonstrates that such an approach is actually beginning to bear fruit. The authors have been targeting peptide deformylase (PDF), an iron metallo-enzyme that removes the formyl group from the obligate N-terminal methionine prior to subsequent protein maturation steps. The essentiality of PDF in bacteria is a controversial subject in that, at least in certain species of pathogenic bacteria, the gene encoding PDF can be inactivated in strains that are mutant in the gene encoding the formyl transferase (*fmt*), which generates N-formylmethionine. However, as the authors point out, such *fmt* mutants grow very poorly *in vitro* and are unlikely to have much pathogenic potential. Previously, these authors demonstrated that the natural product hydroxamic acid actinonin is an antibacterial agent because of its ability to act as a PDF inhibitor (validating both PDF as a 'druggable' target and competition for the metal ion of a metalloenzyme as a viable antimicrobial strategy). Therefore, the authors focused on identifying inhibitors of PDF via a specific chelation strategy using hydroxamic acids.

Findings: The authors have provided a wealth of data, including enzyme inhibition kinetics correlated with antibacterial activity and rationalized by crystallography of enzyme–inhibitor complexes. The authors showed a series of compounds that had IC_{50} (50% enzyme inhibition) concentrations in the nanomolar range versus *E. coli* PDF and similar activity versus the *Streptococcus pneumoniae* version of that enzyme. As a measure of the specificity of these PDF inhibitors the authors demonstrated that none of these compounds had any activity against MMP-7, a matrix metalloprotease. In general these enzyme inhibition data correlated with excellent *in vitro* antimicrobial activity against a range of Gram-positive pathogens, with *S. pneumoniae* being the most susceptible and *Enterococcus faecalis* the least susceptible. Also inhibited were *Haemophilus influenzae* and *Moraxella catarrhalis*. However, wild-type *E. coli* was not susceptible to any of the compounds studied, whereas an *acr* mutant of *E. coli* was quite susceptible, indicating that for some Gram-negative pathogens efflux of the hydroxamic acids renders these bacteria intrinsically resistant. Given this spectrum of activity, PDF inhibitors are most likely to be used to target community-acquired respiratory infections. The authors also studied the emergence of resistance to these compounds and essentially replicated what was seen in their studies on actinonin. In *Staphylococcus aureus fmt,* mutants were identified at about a 10^{-6} frequency, whereas in *S. pneumoniae* and *H. influenzae* mutants in the target itself arose at about a 10^{-9} frequency. Whether or not one is convinced about the long-term viability of hydroxamic acid PDF inhibitors as antibacterial agents, one must admire the well-integrated, comprehensive approach the authors have taken in the discovery of these specific and potent agents. They give hope to the many investigators who are committed to this general approach towards anti-infective drug discovery.

• Sigma B modulates virulence determinant expression and stress resistance: characterization of a functional ***rsbU*** strain derived from ***Staphylococcus aureus*** 8325-4. Horsburgh MJ, Aish JL, White IJ, Shaw L, Lithgow JK, Foster SJ: *J Bacteriol* 2002, 184:5457-5467.

Significance: The most commonly used laboratory strain of staphylococci, 8325-4 (aka RN450; RN6390), contains an 11 base pair deletion in *rbsU*, a gene encoding a positive activator of sigma B, a stress-responsive alternate sigma factor. Sigma B has been shown to have a role in the expression of virulence factors such as coagulase and clumping factor A and regulatory transcription factors SarA and SarS (SarH1). Because 8325-4 has been widely used in studies on the expression of virulence in *Staphylococcus aureus*, the authors sought to determine what affect an intact *rbsU* gene would have on 8325-4.

Findings: The authors constructed a strain, SH1000, which is a version of 8325-4 with a wild-type *rbsU* gene. SH1000 appeared to be marginally more virulent in a mouse skin abscess model. The authors also found decreased levels of expression of α toxin and serine protease, consistent with a decrease in the expression of the accessory gene regulator (*agr*) locus, which the authors observed by reporter gene expression. Unfortunately, the authors did not examine the expression of cell-surface proteins (e.g. protein A and clumping factor), which would have been expected to demonstrate increased levels of expression as a result of decreased expression of *agr* (and may explain the similar levels of relative virulence in the mouse abscess model). It has previously been reported by two independent groups (the laboratories of Cheung and Bischoff) that *sarA* expression is decreased by sigma B expression. This decrease would explain the decrease in *agr* expression, because SarA is necessary for *agr* transcription. In contrast to those findings, the authors of this study report levels of SarA protein analysed by western blot analysis, indicating a SarA-independent role for sigma B. The difference between these studies may lie in the fact that protein levels were measured in this study, whereas the other studies relied on transcription analyses. Alternatively, the antibody used may not be specific for SarA as there are several proteins with varying degrees of amino acid similarity and size to SarA encoded on the *S. aureus* genome.

• Frequency of disinfectant resistance genes and genetic linkage with β-lactamase transposon Tn552 among clinical Staphylococci. Sidhu MS, Heir E, Leegaard T, Wiger K, Holck A: *Antimicrob Agent Chemother* 2002, 46: 2797-2803.

Significance: The finding that resistance determinants can become genetically linked on mobile genetic elements is far from novel, dating to the discovery of resistance transfer factors

by Watanabe. What makes this study noteworthy is the high frequency of multiply resistant strains and the observed linkage of resistance determinants specifying resistance to antibacterial drugs used systemically and agents routinely used as disinfectants and preservatives. Just as the use of antibiotics as growth promoters in animal feed supplements has been waning, we are observing a dramatic increase in consumer products touting their antimicrobial activity. The biocides used for this purpose include mainly quaternary ammonium compounds such as cetylpyridinium chloride or benzalkonium chloride and the fatty acid biosynthesis inhibitor triclosan. In this study the authors examined a series of clinical isolates of *Staphylococcus aureus* and *Staphylococcus epidermidis* for resistance to benzalkonium chloride and determined whether resistance to that quaternary ammonium compound predisposed the bacteria to a multidrug resistance phenotype.
Findings: Of the 61 *S. aureus* strains and 177 coagulase-negative strains examined, 50% were resistant to benzalkonium chloride. When examining the coagulase-negative staphylococci and comparing the benzalkonium-resistant and susceptible strains the authors found higher levels of resistance to each and every drug in a panel of 13, diverse, systemic antibacterial agents among the benzalkonium chloride resistant strains. In most cases the resistance rates were twofold higher or more among the quaternary ammonium compound resistant strains. The authors further studied the degree of genetic linkage between resistance determinants, in one instance mapping the *tetK*, *blaZ* and *qacA/B* genes in close proximity to each other on a plasmid. These genes encode, respectively, a tetracycline efflux protein, penicillinase production and a quaternary ammonium compound efflux pump. The *blaZ* gene in staphylococci is usually associated with the Tn552 transposon, underscoring the potential for mobility of these linked resistance genes. It should be pointed out that the patient population from which these strains were derived (consisting of patients with bloodstream infections, undergoing cancer chemotherapy or with HIV infections) are very likely to have been treated with antibacterials either before or during the study. The continued widespread use of these disinfectants, especially for what can best be considered 'frivolous' applications (e.g. antibacterial pyjamas), compounded with the recent emergence of community-acquired MRSA infections will predictably lead to the widespread dissemination of multiply resistant strains of Gram-positive pathogens. This is likely to happen not just in the hospital setting, but in the community as a whole. This will greatly complicate the current treatment paradigm and eventually force the use of novel, and less efficacious antibacterial drugs as first line therapy.

Food biotechnology

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• ***Lactobacillus plantarum*** MiLAB 393 produces the antifungal cyclic dipeptides cyclo(**L**-Phe-**L**-Pro) and cyclo(**L**-Phe-***trans***-4-OH-**L**-Pro) and 3-phenyllactic acid. Ström K, Sjögren J, Broberg A, Schnürer J: *Appl Environ Microbiol* 2002, 68:4322-4327.
Significance: This paper describes the characterisation of novel antifungal compounds produced by a *Lactobacillus plantarum* strain isolated from grass silage.
Findings: Antifungal activity produced by food-grade microorganisms has been studied extensively in the past. These studies have led to the identification of a number of low molecular weight molecules, mostly organic acids, that display this activity. This paper describes the identification and characterisation of novel antifungal compounds produced by *L. plantarum.* The compounds found were cyclo(L-Phe-L-Pro), cyclo(L-Phe-*trans*-4-OH- L-Pro) and 3-phenyllactic acid (L/D isomer ratio, 9:1). This is the first report on the production of antifungal compounds of this nature by lactic acid bacteria and, moreover, is the first report to clearly establish the antifungal activity of these compounds. These findings could open avenues for the inhibition of fungal spoilage of fermented foods by using starter bacteria that produce these or similar antifungal compounds during fermentation.

• ***Escherichia coli*** O157 and non-O157 isolates are more susceptible to **L**-lactate than to **D**-lactate. McWilliams Leitch EC, Stewart CS: *Appl Environ Microbiol* 2002, 68:4676-4678.
Significance: *Escherichia coli* O157 and non-O157 isolates are more susceptible to L-lactate than to D-lactate. This finding will have a major impact on the choice of lactic acid bacteria as probiotics or for the production of lactic acid as preservative.
Findings: D-Lactic acid and L-lactic acid were compared for their bacteriocidal effect on *E. coli* O157 and several other isolates. About 50% more D-lactic acid was needed than L-lactic acid to reach the same killing efficiency. Both lactic acid isomers had exactly the same effect on the transmembrane pH gradient, meaning that the differences observed cannot be explained on the level of the (low) internal pH. Apparently, *E. coli* has a, yet unknown, mechanism that confers tolerance to D-lactic acid, the natural fermentation product, over L-lactic acid.

Chemical biotechnology

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• Development of a donor-acceptor concept for enzymatic cross-coupling reactions of aldehydes: the first asymmetric cross-benzoin condensation. Dünkelmann P, Kolter-Jung D, Nitsche A, Demir AS, Siegert P, Lingen B, Baumann M, Pohl M, Müller M: *J Am Chem Soc* 2002, 124:12084-12085.
Significance: An enzymatic asymmetric mixed benzoin synthesis has been presented for the first time, which has significant advantages over previous chemical methods.
Findings: The thiamin diphosphate dependent enzymes benzaldehyde lyase (BAL) and the benzoformate decarboxylase (BFD) H281A variant were compared for their ability to catalyse the specific coupling of various donor and acceptor substituted benzaldehyde derivatives in benzoin syntheses. It was found that these enzymatic reactions could be highly specific for the production of mixed benzoins depending on the particular aromatic substitutions and that certain substitutions were reliably either the donor or acceptor molecules in the enzymatic cross-coupling reaction. Using this enzymatic system it was possible to prepare mixed benzoins at over 99% conversion, selectivity and enantiomeric excess on a gram scale.

• Use of enzymes deactivated by site-directed mutagenesis for the preparation of enantioselective membranes. Skolaut A, Retey J: *Angew Chem Int Ed* 2002, 41:2960-2962.
Significance: Site-directed mutagenesis has been used to eliminate the catalytic activity of the enzymes histidine ammonia lyase (HAL) and phenylalanine ammonia lyase (PAL). Both inactive enzymes were subsequently immobilised onto artificial membranes and used to facilitate enantioselective transport of L-histidine and L-phenylalanine, respectively.
Findings: Enantioselective membranes are of interest in that they can facilitate the transport of chiral molecules, thereby effecting a kinetic resolution process. Previous work had shown that enzymes can be converted to non-catalytic chiral receptors by depriving the enzymes of their cofactors. In the current report, the authors have rendered two lyase enzymes inactive by removing catalytically essential active-site residues in each case. After screening a number of site-specific variants, the PAL Y109F and HAL E414A proteins were selected on the basis that they retained their binding activity, but had lost almost all catalytic activity. The two mutants were immobilised into poly(dimethylsiloxane) polymer films between the surfaces of two alumina membranes, effectively forming a membrane sandwich. The uptake experiments were performed by placing the membrane, containing the inactivated enzyme, between a solution of the racemic substrate and a substrate-free buffer solution. At certain time points, the enantiomeric composition in the two cells was determined by chiral HPLC, allowing the ratio of transported L- and D-enantiomers to be determined (termed the selectivity coefficient, SF). At 0.1 mM concentration of DL-phenylalanine, the PAL Y109F mutant exhibited a maximum SF of 2.5 in favour of the L-enantiomer. By comparison, the HAL E414A mutant exhibited higher selectivity with the SF value reaching a maximum value of 13.3. Both systems showed a concentration dependence of the SF values. Control experiments using membranes without encapsulated enzyme showed no enantioselectivity.

• New natural product families from an environmental DNA (eDNA) gene cluster. Brady SF, Chao CJ, Clardy J: *J Am Chem Soc* 2002, 124:9968-9969.
Significance: Uncultured soil microbes are a potentially rich source of new enzyme activities and biologically active small molecules. Here, a biosynthetic gene cluster from DNA isolated from soil (eDNA) that produces derivatives of long-chain *N*-acyltyrosine antibiotics when cloned into *Escherichia coli* was investigated.
Findings: A clone was isolated from an eDNA library that not only produced *N*-acyltyrosine antibiotics but two additional families of clone-specific small molecules. The new molecules were analysed: one was found to be a decarboxylated *N*-acyltyrosine derivative (eneamide) and the other underwent a further NH to O substitution to produce the enol ester. The eDNA was analysed and found to contain a secondary metabolite gene cluster of 13 open reading frames. The roles of each reading frame were investigated by gene knockout and the genes for the production of the new metabolite families were identified including the decarboxylase and *N,O*-acyltransferase. The role of the remaining genes in the cluster was inferred from BLAST searches and this allowed a full biosynthetic scheme to be proposed.