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Highlight

Metabolic engineering, new antibiotics and biofilm viscoelasticity

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In the following highlight we refer to a number of new advances in the field of Biotechnology that address issues relating to the synthesis of new antibiotics, new biocatalysts and matrices in biofilms.

Biofilms and pathogens

In the article by DiStefano and colleagues (2009), biofilms of *Staphylococcus aureus* and *S. epidermidis* strains (two reference strains and two ocular isolates) were analysed in terms of their viscoelastic properties, along with the characterization of some virulence traits and biofilm structures. These bacteria are relevant pathogens that can cause chronic infections, associated with their ability to establish a biofilm on the surface of medical devices (implants, catheters) or other materials such as contact lenses. The results give an idea of the rheological properties of these biofilms and their behaviour as viscoelastic materials, showing also how biofilms formed by closely related strains may have significantly different characteristics and physical properties. The ultimate causes for these differences offer a valuable focus for future work, since they may shed light on the various survival and adaptation strategies adopted by related microorganisms.

This work by DiStefano and colleagues (2009) opens the door to transversal and multidisciplinary research in the biofilm field, whereby the fields of physics, bacterial physiology and genetics can join forces to provide novel and important information on the structure and stability of microbial biofilms. As the authors point out, therapies combining antimicrobial agents with compounds capable of altering the viscoelastic properties of the biofilm could prove highly effective to combat biofilm-associated chronic infections. Furthermore, biotechnological applications involving biofilms, such as membrane or granular sludge bioreactors, could benefit from this knowledge. Being able to predict how the biofilm will behave under the flow conditions imposed in the bioreactor or when shear forces change might result in a significant improvement in performance.

Moschioni and colleagues (2009) from Novartis Vaccine and Diagnostic have reviewed the field of adhesins within a collection of important human and animal pathogens grouped under the genus *Streptococcus*. These microorganisms cause pharyngitis, scarlet fever, as well as severe skin infections and invasive infections (endocarditis, necrotizing fasciitis etc.). They are also primary colonizers of the surface of teeth and are abundant in dental plaque. The authors emphasize that bacterial adherence to host tissues represents a critical step in pathogenic processes and is usually mediated by bacterial surface-exposed proteins. Adhesins can be either single molecules or ordered structures such as pili or fimbriae. Interactions often involve binding to host extracellular matrix components and cell surfaces. Streptococcal adhesins have been grouped into families based on the substrate they bind to, although some redundancy tends to occur. The authors discuss examples of adhesins that bind to fibronectin, fibrinogen, lamini, AgI/II family proteins and others such as choline-binding proteins. Fibronectin-binding proteins have a modular structure with a C-terminal cell-wall-anchoring motif and a short positively charged tail. In this C-terminal region short repeats of 30–40 residues can be found. This type of repetitive motif has also been found in some other adhesion family members and may represent a distinctive feature of adhesins since these sequence repeats are often found within *Pseudomonas putida* and *P. fluorescens* adhesins (Espinosa-Urgel and Ramos, 2004; Hinsa and O'Toole, 2006; Yousef and Espinosa-Urgel, 2007). The authors go on to discuss the interest in identifying small molecules that may interfere with one or more of these adhesins and their potential to be incorporated into dentistry products as

inhibitors of plaque formation. They also discuss the potential of pili and fimbriae to generate vaccines and suggest research on potential applications of adhesins in biotechnological applications. The review also takes advantage of the extensive knowledge of the genomics of these microorganisms and the previous characterization of many different species in the genus, allowing insight into the set of well-studied adhesins, which is complemented by a very exhaustive citation of previous work that facilitates further exploration by the reader.

New antibiotics

With the increased prevalence of multidrug-resistant strains of bacteria, cutting edge research is required to enhance our understanding of antibiotics, as well as the development of new forms of antimicrobials. Three articles that are featured in this issue provide timely insight into both of these topics. Grosse and colleagues (2009) identify two novel esterases that could be used to optimize the biosynthesis of levofloxacin (LFC) – a third-generation antibiotic that is used clinically to treat a number of infections, including multidrug-resistant tuberculosis. The two esterases, BcEST and TtEST, act on a key intermediate in LFC biosynthesis in an enantioselective manner, and may therefore be used to resolve racemic mixtures, providing a more green and chemo-enzymatic route to LFC production. With the aim of improving the stability of BcEST, the authors carried out directed evolution. After a two-step mutagenesis screen for mutants exhibiting intact activity at higher temperatures, and the analysis of close to 7000 strains, they showed that the apparent melting temperature (T_m) of the BcEST enzyme could be increased by up to five degrees. These results were then compared with the performance of TtEST, which was isolated from a thermophilic microorganism, showing that the naturally occurring biocatalyst outperformed the experimentally evolved BcEST mutants. As such, their results also show that genome mining within thermophiles may provide a cost-effective approach for the discovery of new thermostable biocatalysts.

The article by Deegan and colleagues (2009) also has important implications in the fight against multidrug-resistant bacteria, although, rather than concentrating on traditional antibiotics, the work is focused on an unusual type of antibiotic called lantibiotics. These antimicrobial peptides are of particular importance due to the fact that they exhibit activity against multidrug-resistant pathogens at minute concentrations. By creating a set of 16 variants of a two-peptide lantibiotic, named lacticin 3147, the work clarifies a number of aspects about the determinants of antibacterial activity. While previous work has suggested that the overall charge of a lantibiotic may be important to activity, the current results show that changes made to the peptides impact antimicrobial activity in a location-specific manner. This is particularly apparent for one of the variants of the lacticin 3147 peptides, which was made to be uncharged while still retaining bioactivity. Notably, when assessing the activity of the variants, it was found that one exhibits heightened antibacterial activity. This result is significant in that it is the first reported case in which the activity of a lantibiotic has been increased. The challenge raised by subsequent results is that, although the peptide is twice as active by itself, the normal synergy that occurs when both peptides are present is greatly reduced. Despite this difficulty, the work calls for new research focused on the optimization of such peptides to further increase their efficacy. Lantibiotics are particularly relevant to solving the problem of the rapid rise of multidrug-resistant bacteria, providing a quick and versatile tool for the development of new and powerful antibacterial compounds.

Another interesting group of molecules that are relevant for pharmaceutical research and drug development are β-peptides or mixed β,α-peptides that contain β-amino acid residues – naturally occurring products that exhibit high biological activities. While common α-peptides are rapidly degraded *in vivo* and *in vitro* by a multitude of enzymes, peptides containing β-amino acid residues show a high level of resistance to proteolytic attack, making them ideal candidates for new antibiotics and other pharmacologically active compounds. In the report presented by Heyland and colleagues (2009), both factors are considered as they describe the development and characterization of whole-cell biocatalyst systems for obtaining β,α-peptide L-carnosine. The use of whole cells as industrial biocatalysers can considerably reduce time and material costs associated with alternative chemical synthesis procedures, although usually the technique may be limited due to reduced activity or the generation of unwanted byproducts. The authors examine differences in the production of β,α-peptides (DmpA and 3-2W4 BapA) using enzymes versus the use of recombinant hosts (*Escherichia coli* and *Pichia pastoris*), thereby comparing the use of crude extracts versus whole cells systems. They selected *E. coli* whole cells with recombinant DmpA as the most promising production method and, with this system, performed further optimization studies by varying the cell density, temperature, pH, induction kinetics and substrate concentration. Furthermore, they also checked the stability and the efficacy of the process in both batch and fed-batch cultures, obtaining satisfactory rates in both conditions, together with scarce production of undesirable secondary metabolites. These results could be applied to the production of new peptides with useful biological activities although further efforts are required

in order to optimize the expensive substrate needs and to develop efficient new methods for product purification.

Metagenomics and biocatalysis

A recent Special Issue of *Environmental Microbiology* outlined a number of approaches that have been developed for the recovery of DNA from a given niche during metagenomic analysis. Cloning of 'environmental' DNA without the need to cultivate the source microorganism is a tremendous advantage in spite of the fact that a number of biases in cloning and/or expression have been identified (Aharoni, 2009; Ferrer, 2009). One of the most relevant advantages of massive DNA cloning is the increased possibility of discovering enzymes with novel properties. In the current issue of *Microbial Biotechnology*, two research articles deal with the recovery of relevant new enzymes from the metagenomic point of view. In one of the articles, Vieites and colleagues (2009) screen a metagenomic library constructed from the gut of the earthworm *Aporrectdea caliiginosa*. During the experimental process, the earthworm was fed with cellulosic compounds in order to favour the growth of microbes that are able to attack esters. Upon detailed screening, the authors describe an esterase, called 3A6, that exhibits unusual activity as a carboxyl esterase and a feruloyl esterase, which is significantly different from the known function of other proteins homologous to 3A6. Using docking methods, the authors construct a model of the enzyme based on the available 3D structures of other carboxyl esterases. The model identifies three residues (Ser-143, Asp-273 and His-305) as potential catalytic sites, which are confirmed upon the generation of point mutants that lose activity. The model is then examined to define 'areas of influence' regarding substrate specificity and substrate preference. These regions comprise triads or dyads made of residues 109–111, 315–317 and 281/282. The authors have performed exhaustive site-directed mutagenesis of these regions followed by substrate discrimination assays. Mutants in these residues are often associated with an increased preference in the mutant variant for carboxyl esterase versus feruloyl esterase activity, while a correlation between substrate preference and activation energy exists. One of the most remarkable findings is that a 34-amino-acid residue loop deletion leads to a variant that retains carboxyl esterase activity while completely losing feruloyl esterase activity. Vieites *et al.* discuss how feruloyl esterase activity may be the result of selective pressure through nutritional/environmental situations that demand for a broader range of substrates at the expense of specificity. In this regard the potential for regulators to interact with a variety of effector molecules; i.e. XylR (Garmendia *et al.* 2009) and XylS variants (Brautaset *et al.*, 2008) have been described. Although the plasticity of the enzymes and regulators reflects their evolutionary capacity, it seems unlikely that the changes can occur in such an immediate fashion that the response can account for the temporary nutritional variation in the microbial status. The research article by Vieites *et al.*, together with the instructive review on glutamate racemase by Fisher (2009), advocates the relevant role *Microbial Biotechnology* can play in the diffusion of important biocatalysis information.

Fernández-Álvaro and colleagues (2009) also uses metagenomic libraries to search for relevant new enzymatic activities. In particular, the author screens one such library for the hydrolysis of ethyl esters within racemic phenylalkyl carboxylic acids. The elegant pH indicator assay used was further refined to obtain enantioselective enzymes, which were then utilized to obtain excellent optically pure chemicals. This work provides an example showing how the search for specific activities provides an excellent approach for the advancement of the use of enzymes and microbes in the synthesis of chemicals.

Another innovative approach in the design of biocatalytic processes is the external control of the reactions. In this issue, Kraus and colleagues (2009) present an attractive review on the use of LOV (Light, Oxygen, Voltage) domains for the construction of photoactive-controlled chimeric proteins. Furthermore, the authors develop the use of this widely used mechanism for the construction of functional hybrid regulators that can be easily controlled by light. The advantages of photoreceptors as external inducers include limited cell damage and, most importantly, precise control in the timing and the extent of the signal effect, while additionally, these new hybrid regulators could be potentially used within a broad range of organisms as photochromic proteins are present in numerous biological processes. In the past, this approach has been successfully used in the construction of chimeras between structurally related proteins. For example, Stricland and colleagues (2008) placed the *E. coli trp* repressor domain under the control of the LOV domain from *Avena sativa* phototropin 1. The main technical difficulty associated with the use of these constructs when connecting unrelated proteins is that it is often difficult to ensure that the domains are fused at the right positions. To solve this problem, the authors propose the use of statistical coupling analysis (SCA) to identify networks of co-evolving amino acids in different proteins, which allows the location of potential surface allosteric sites connecting distant protein domains. Using this approach, they designed fusions between a LOV domain to the DHFR biocatalytic region, generating enzymes that catalyse the reduction of 5,6-dihydrofolate under light control. The mechanism proposed opens new avenues in the research of effective and inexpensive regulators for biocatalysis. Future research will be required to improve the efficacy of the process and to discover potential uses for modulating enzyme specificity.

Enhanced vitamin production

Production of commercially significant metabolites that are important human nutrients is a vital field of microbial biotechnology (Sánchez and Demain, 2009). The amplification of commercial production processes is of fundamental importance as increased yields translate into positive public and financial outcomes. Recently in *Microbial Biotechnology* Biedendieck and colleagues (2009) published a fascinating research article which detailed the metabolic engineering of *Bacillus megaterium* to increase the production of cobalamin (vitamin B_{12}). They used the cobalamin production system of *B. megaterium* to assess various genetic optimization strategies including plasmid and genome-based overexpression of genes, enzyme modification for increased stability and manipulation of regulatory genes. The authors report that all of the adjustments could lead to increased production of cobalamin, with some of the manipulations, such as the overexpression of cobalamin pathways, leading to a near 40-fold increase in B_{12} production. Follow-up experiments included the use of an antisense RNA strategy to reduce the flow of metabolites along an alternative route and removal of the riboswitch inhibitory structure located upstream of the main B_{12} operon. Again, these latter modifications were shown to increase cobalamin production in *B. megaterium* when used individually. Interestingly and somewhat unfortunately, experiments using combinations of the above manipulations, while able to augment the intracellular cobalamin concentrations, showed compromised cell growth because the alterations placed the bacteria under metabolic stress. This research emphasizes the importance of a systematic approach in the implementation of strategies to improve nutrient metabolite yield for large-scale production systems and suggests that a more subtle approach may garner a better final result.

Biodegradation

Polycyclic aromatic hydrocarbons (PAHs), considered common examples of persistent organic pollutants (POPs), have been reported to be present in many environments. As a result of their low water solubility and strong sorption to soil particles (mainly to organic matter and clay fractions), these compounds can exhibit extremely long half-lives exceeding 1000 days for some PAHs, as benzo[a]pyrene in soils and sediments. Since PAHs occur in the environment as complex mixtures, behaviour and capabilities of bacterial consortia need to be elucidated. Moreover, it is well known that in some cases, low-molecular-weight PAHs can inhibit or restrict the biodegradation of heavier compounds (reviewed by Ri-He *et al.*, 2008). Despite the fact that PAHs are subjected to different abiotic processes, once they have been released into the environment (volatilization, photooxidation), it is assumed that biodegradation is the main way that these compounds are removed (Lotfabat and Gray, 2002). Thus the great interest that exists for the identification of the mechanisms used by microorganisms to degrade these persistent chemicals is not surprising. Indeed, a considerable number of bacteria with PAH-degrading capabilities, either growth-linked reactions or cometabolic processes, have been isolated and characterized. Since the biodegradation of low-molecular-weight PAHs (especially naphthalene) is well documented, future efforts must be aimed towards studying the biodegradation of high-molecular-weight compounds (HMW), that is, those that contain more than three benzene rings. Along these lines, the current issue of *Microbial Biotechnology* features a article by Kanaly and Harayama (2009), in which the authors offer us a new and a comprehensive study of HMW-PAH biodegradation. Continuing along the lines of previous publications (Kanaly and Harayama, 2000), these authors present us a magnificent overview of the achievements attained during the past 20 years, not only for pyrene and fluoranthene, but also for benzo[a]anthracene and benzo[a]pyrene. Since the first reports published in 1975 by Gibson and Barnsley about biodegradation of the latter two compounds cited above, advances have been made due to the isolation of new PAH-degrading microorganisms as well as in-depth investigations of previously known microorganisms, such as *Mycobacterium vanbaalenii* PYR-1, which is one of the most relevant PAH degraders. In fact, the metabolic pathway used by this strain for the biodegradation of fluoranthene has been recently elucidated. Novel metabolites and enzymes have been detected and identified, and new pathways have been proposed in the last two decades. In connection with this, methanogenic biodegradation of PAHs has been recently postulated (Dolfing *et al.*, 2009). Furthermore, development of a number of new metabolomic, genomic and proteomic technologies has contributed enormously to these findings. In summary, the article by Kanaly and Karayama is an invaluable source of information for those who wish to deepen their knowledge of HMW-PAH biodegradation.

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