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From the test tube to the Environment –and back

V. de Lorenzo, Dietmar Pieper and J. L. Ramos

Introduction: Molecular Biology meets Environmental Microbiology

Many consider 1972 as the year where the still nascent field of Plasmid Biology met the also embryonic realm of Environmental Microbiology for the first time. By that time, Al Chakrabarty had put forward its now renowned –and extremely controversial Patent US 4,259,444 on the genetic improvement of *Pseudomonas* for oil biodegradation (1981). Although the strains at stake were not ultimately as useful as initially claimed and the genetic engineering tools available in the 70s were very limited, the case elicited the imagination of a selected group of by then young researchers that were eager to use recombinant DNA technology to address a large number of scientific issues, as well as to contribute to solve many of the most burning problems of mankind e.g. environmental pollution. However it took many more years to provide a solid basis to this endeavour and to have a constellation of scientists taking over the challenge of exploring the still *terra incognita* at the boundary between Molecular Biology and Microbial Ecology. Breakthroughs over during this period of time included a series epoch-making papers from the Laboratory of Ken Timmis (Lehrbach *et al*., 1984; Ramos *et al*., 1987; Rojo *et al*., 1987; Mermod *et al*., 1986) which showed that genetic engineering could indeed be instrumental to improve the catalytic abilities of environmental bacteria and thus have a way of increasing biodegradation of toxic chemicals in polluted sites. These papers were echoed in the agricultural side by the work of Steve Lindow and his epiphytic ice-minus *Pseudomonas* able to protect strawberry fields from frost (1987). In either case the decisive step was not so much the genetic engineering of the agents of interest (which were often successful in the Laboratory), but their actual release into the target sites to do the job. Whether by virtue of by necessity, Molecular Biologists and Bacterial Geneticists had to face the overwhelming task of moving bacteria from the comfortable life of the Laboratory back into their natural *red in tooth and claw* habitats. It was soon realized that this was barely possible without a profound understanding of the ecological background of such interventions-to-be. At the same time, the possibility of deliberately releasing genetically engineered microbes (GEMs) for environmental cleanup raised all alarms on the (real or imaginary) risks associated to putting in biological agents that had not existed in Nature before. By the end of the 80s such developments put the focus on Microbial Ecology and Environmental Microbiology, two related fields that until then had been largely marginal to frontline scientific research.

The birth of a new scientific and biotechnological discipline

It was in this context of hope and fears that a large –and to some extent foundational meeting on *Molecular Microbial Ecology* was called in 1989 upon the German city of Braunschweig by Ken Timmis and Rob Steffan to discuss the state of affairs, identify research topics and draw up an agenda of the field for the next 20 years. It is remarkable how many of the topics pinpointed there and then became veritable attractors of much environmental research during the decades to come: genetic and biological containment, non-culturable microorganisms, horizontal gene transfer, biosensors, bioavailability, bioremediation, molecular tools, community structure and many others. Metagenomics (and all the other -*omics* that followed suit) had to wait another 10 years until the wet tools, fast DNA sequencing methods and bioinformatic resources were in place to access the immense genetic and enzymatic reservoir of the microbial world. This endeavour reached one peak in the *Sorcerer II* global ocean sampling expedition run by C. Venter for several years in the past decade (Rusch *et al*., 2007) and which continues today through the systematic exploration of microbial niches of every imaginable origin.

Now is thus the time of taking stock of what has been going on –and is still running in Environmental Microbiology since the mid-80s and reflect on what questions have been solved, which new ones have emerged and what elements are necessary to deal with them –technically and conceptually. With these ideas in mind, we set out to organize a relatively small workshop in the Renaissance city of Baeza in Andalucía to tackle these issues[a]. And what could be more adequate than using Ken Timmis' extraordinary career as the background and blueprint for developing a meeting program that could faithfully reflect the progress in the field. This Special Issue of *Env Microbiol* is mostly shaped by papers submitted by participants of this meeting –many of them former and current collaborators of Timmis not only in the hope of mapping where the subject stands at the moment, but also to pay a tribute to the extraordinary leadership that Ken has exerted over the years in the same territory. Readers may take this collective issue as a piece of what in the best German academic tradition is called a *Festschrift* i.e., a compilation of original papers by the honoured Academic's close colleagues which is published on the occasion of a notable career anniversary.

Pseudomomonas putida as an engineer-able whole-cell catalyst

One of Ken's lasting contributions has been his vision of *Pseudomonas putida* as the one optimal workhorse (what today would be called a *genomic chassis*, [Silva-Rocha *et al*., 2011]) for industrial and environmental biotechnology. The so-called KT2440 strain of *P. putida* (KT = Ken Timmis) is considered one of the safest and most secure hosts for foreign gene cloning, as it was certified as back in 1981 by the Recombinant DNA Advisory Committee (RAC) of the NIH as the first Host-Vector Biosafety (HV1) system for gene cloning in Gram-negative soil bacteria. Later sequencing of its genome revealed a conspicuous lack of any recognizable virulence factor or pathogenesis trait, even as an opportunistic pathogen. This makes *P. putida* KT2440 a safe organism of choice both as host of biocatalytic reactions and as an agent for *in situ* bioremediation. It thus comes a no surprise that a number of articles of this issue deal with the fundamental biology of this remarkable microorganism. One matter that has kept various Laboratories busy in the last decade is how *P. putida* makes choices between various possible C and N sources, but the picture gets more intricate by the day. Seminal observations on how glucose and amino acids (See Rojo, 2010 for a review) down-regulate expression of the genes for biodegradation of m-xylene by *P. putida* mt-2 (i.e., KT2440 strain carrying the TOL plasmid pWW0) have evolved into a quite complex regulatory scenario where not only nutrients, but also many other physiological conditions act as signals to opt for the expression or not of given sets of catabolic genes. In this context, Fonseca *et al.* (2012) show that the effect of the two sRNAs (CrcY and CrcZ) that bind to and sequester the Crc RNA-binding protein (the main mediator of catabolite repression in Pseudomononads, [Moreno *et al*., 2010]) is influenced by temperature. That sRNA structure and functionality is very sensitive to thermal changes is not unknown, but the beauty of the system relies, in this case, on how such sensitivity can afford merging biochemical signals with physical inputs in the same lot. As shown also in the article by Hernández-Arranz (2012) the number of mRNAs that have been identified as Crc targets in *P. putida* is growing. Intriguingly, it is not unusual that such targets include both the transcriptional regulator of a given catabolic pathway (e.g. BenR, AlkS) as well as one or more of the protein products (e.g. enzymes, transporters) that are controlled by them. The reason for such a *regulatory overkill* based on the post-transcriptional control of various components of the same expression motif is uncertain and deserves further studies. Silva-Rocha *et al*. (2012) present some fascinating aspects on the role of the global regulator Integration Host Factor

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(IHF); the first one is that although deletion of the *ihfA* affects the expression of a number of genes to a certain extent, its deficiency does not lead to a general failure in growth capabilities and matter of factly metabolic flux analysis revealed that IHF does not affect central carbon catabolism; however it has a significant effect in the control of a number of genes encoding surface-related functions, ATP synthase component and the levels of the RNA polymerase alpha subunit. IHF has been suggested a role in setting the conditions to prepare the physiology of the cell in the transition from the logarithmic growth phase to the stationary phase.

Comparing *P. putida* with its relatives

Those who believe the celebrated quote of J. Monod *'what is true of E. coli must also be true of elephants'* might be shocked to learn how different are the strategies *P. putida* adopts for dealing with otherwise similar metabolic and regulatory problems. A remarkable case is presented by Chavarria *et al.* (2012) regarding the physiological roles of the metabolic flux sensor protein called Cra (for catabolite repression / activation). While in *E. coli* this proteins seems to be a global regulator of metabolic activity through its binding to the glycolytic intermediate fructose-1,6-bisphosphate, which is then translated in activation and repression of a large number of cellular functions (XX), its role in *P. putida* is largely limited to control fructose uptake and its cross-talk with the so-called N-related branch of the phosphoenolpyruvate-dependent phosphotransfer system or PTS. This regulatory *exaptation* of the Cra protein of *P. putida* has been observed also in other global regulators of this bacterium such as the Crp protein (Perrenoud *et al*., 2005) and the integration host factor, IHF (Silva-Rocha *et al*., 2012). In other cases, *E. coli* and *P. putida* may consume the same C source, but some features of the process are entirely different. Escapa *et al*. (2012) report a number of striking characteristics of glycerol utilization by *P. putida,* the most conspicuous of which being the extraordinarily long lag phase that cells undergo before taking off at very rapid growth rates. This work shows this phenomenon to rely on the GlpR repressor, the inhibitory action of which might be relieved only upon accumulation of a metabolic signal of energy status or by the external addition of octanoate. This is an interesting detail that may help to expand the use of *P. putida* for biotechnological applications of glycerol, a bulk residue of biodiesel production. Comparisons of *P. putida* with other, often very related bacteria, produce also considerable dividends both in fundamental knowledge and prospective practical uses. The report by Duque *et al.* (2012) abounds in this approach by comparing determinants of adhesion to both biotic and abiotic solid surfaces of *P. putida* and *P. aeruginosa*. Much of this work is based on the extraordinary resource built over the years by these authors which involves a large stock of mapped insertion mutants of *P. putida* as well as other large collections resulting from random mini-Tn*5* mutagenesis. The result of this this study shows how a core of 8 adhesion-related genes are shared by both strains, other specific functions notwithstanding for each bacterium. Cell-to-cell communication is a fascinating area of research and one of the most well known phenomenona is that of quorum sensing. The classical acyl- homoserine lactone is present is some but no all of the *P. putida* strains and relatives, but ways to communicate via volatile fatty acids and other signals have been recently proposed (Fernández-Piñar *et al*., 2012). In this issue Bobadilla *et al*. (2012) show that two strains of *Pseudomonas*, B13 and *reinekei* MT1, use protoanemonin (Blasco *et al*., 1996) as a quorum-sensing signal, a role that has to be added to the already described role of being an antibiotic. This raises a very relevant point: what is an antibiotic and which is (are) their role in the natural environment?

The amazing creativity of environmental bacteria

In reality, *P. aeruginosa* is a world of its own and its astonishing capacity of fast adaptation to all sorts of habitats is both a serious clinical problem and a fascinating evolutionary case. The article by Klockgether *et al.* (2012) illustrates this feature of *P. aeruginosa* biology by comparing the genomic organization of two isolates from cystic fibrosis patients. The clear divergences between strains that

inhabit otherwise similar niches tell us about the effect of the non-homogeneous physico-chemical structure of the lung on genetic and genomic bifurcations on initially clonal populations. And they also say something regarding the evolutionary creativity of this bacterial genus for dealing with new environments. Stunning cases in this respect include the ability of some Pseudomonads to use compounds that are extremely toxic for us and for much of the biological world as nutrients. Luque-Almagro *et al.* (2012) document some features of the genome of a *P. pseudoalcaligenes* strain that not only tolerates the presence of cyanide in the medium, but also uses it happily as nitrogen source. It is puzzling that the interest on such cyanide-eating bacteria has barely gone beyond the realm of *Pseudomonas*' aficionados. Yet, one does not need to go to such nutritional extremes to appreciate the immense biochemical power of bacteria of this type. One of the long lasting tenets in biodegradation has been that chlorocatechols are metabolized through an *ortho*-cleavage pathway whereas methyl-catechols do so *via* a *meta*-cleavage mechanism. But it happens that (inter alia) *P. putida* strain GJ31 grows on chlorobenzene using a *meta* pathway with chlorocatechol 2,3-dioxygenase (CbzE) as a key step. This noteworthy enzyme has received since much attention and Schmidt *et al.* (2012) explain their efforts for understanding the molecular determinants that cause substrate specificity of such CbzE product. Forward engineering of functional substrate binding sites in enzymes remains as one of the *holy grails* of industrial and environmental engineering and the last few years have witnessed both spectacular advances and not less spectacular failures in this endeavour. Still, the aerobic world where most Pseudomonads thrive is only a minute part of the much wider catalytic abilities available in the global microbiota. The technical difficulties for studying biodegradation of toxic chemicals under anaerobic or anoxic conditions, the much slower growth of the bacteria at stake, have postponed until recently the much awaited encounter between molecular biology and the life-without-oxygen. Fortunately, genetically manageable model systems have emerged in the last few years that allow a fresh look to otherwise challenging metabolic questions raised by anaerobic physiology. The details provided by Juarez *et al.* (2012) on the pathways for the anaerobic catabolism of 3-methylbenzoate and *m*-xylene by *Azoarcus* sp. CIB exemplify well how the same chemical problem is solved through entirely different biochemical strategies depending on whether oxygen is available or not.

Bioremediation was a very relevant part of the Baeza meeting and two papers dealt masterfully with pollution by oil spills, either in artificial laboratory-polluted sites (Chronopoulou *et al*., 2012) or in a natural setting (Acosta-González *et al*., 2012). In this latter case, a relevant study on microbial degradation under anaerobic conditions in sediments polluted by the sinking of the Prestige is presented. It is frankly astonishing to see how biodiversity and the removal of pollutants varies within a few millimetres of the sediment from the most oxic zone, where aerobes are predominant, to other zones where nitrate respiring and iron respiring-microbes are found. Oil pollution has also a very significant effect on the population of phototrophic microbes, which leads to changes in the trophic chain. This world remains still vastly unexplored. However, new approaches were presented on to go deeper and deeper into the elucidation of what is there and what their role might be. The advances in second-generation DNA sequencing technologies is revolutionizing our capacity to identify microbes, and in-depth analyses using a wide series of algorithms to determine polymorphism can help immensely to resolve the number and abundance of individual clonal complexes of dominant species in polymicrobial communities (Davenport and Tummler, 2012). This combined with the appropriate tools to explore unexplorable sites such as deep marine pelagic zones is revealing that microbial biodiversity is wider that we can ever imagine, and that it does not only represent the most diverse microbes in nature, but probably, from the mass point of view, the most abundant (Smedile *et al*., 2012). If metagenomics has provided an enormous amount of DNA sequences, new approaches are upcoming to provide further details on the corresponding gene products.This can be very relevant not only in the identification of microbes based on MALDI-TOF, as shown by Christi-Oleza *et al*. (2012), to identify members of the genus *Ruegeria*, but the new generation of proteomics tools is beginning to

see the light and revealing new aspects of the live of microbes. This is reviewed by Jean Armengold (2012) who craftly explores how hybrid-high resolution mass spectrometry is opening new perspectives in the microbiology world. In fact, some discoveries are challenging certain *dogmas* in the field, for instance in microbes, initiation of protein does not occur predominantly from ATG codons; the non-canonical codons influence the production of proteins or the fact that a gene may encode more than one gene product. This was well established for viruses, but it was not so apparent for microbes, but evidences are mounting that genomes have to be screened to define the roles of proteins.

Animal and human bodies are "environments *per se*", Ferrer *et al*. (2012) analysed the structure of microbial communities in the distal gut of lean and obese adolescents, and although the study is based on a very limited number of samples, it seems that Firmicutes are dominant in the gut of obese youngters versus dominance of Bacteriodetes in the case of lean people. Whether microbes will be able to control our weight is still matter of speculation, but the miracle diet might actually be the microbial diet!

Vaccines have been linked to environmental microbiology since the seminal work of Pasteur. Carlos Guzman, who has collaborated very closely with Ken Timmis in the last decade, presents an important paper on development of attenuated vaccine strains of *Bordotella bronchiseptica* (Yevsa *et al*., 2012), an important pathogen that causes a number of veterinary respiratory syndromes in food-producing animals. These findings can help to overcome, at least partly, the economical losses. Vaccine strains protect the animals against colonization by wild-type counterparts and are designed so that the virulence character cannot revert through recombination. This represents an advance of paramount importance in the field (Libanova *et al*., 2012; Vogel and Claus, 2011; de Gregorio and Rappuoli, 2012).

Conclusion

What general take-home lessons can be distilled from these papers and the Workshop as a whole? Let us outline at least 3 inferences from both the articles contributed below and the many lectures, short talks and discussions that took place in Baeza. **The first** is the growing paradox between the huge amount of data that can de derived from current -*omics* techniques applied to environmental settings, and the comparatively slower rate of producing authentic knowledge about them. Mistaking data by true knowledge is one of most frequent errors of our time, and not easy to overcome. Whether we like it or not, handling data is the business of Information Technologies, not of traditional *wet* Biology and there is much to do for merging Environmental Microbiology with computation beyond being a simple aid for organizing results. Some even argue that bacteria are themselves computers making computers. At the end of the day, microbial systems display an amazing ability to process environmental signals and to find solutions to extremely intricate adaptation problems. We have been too fascinated with cataloguing the *hardware* of environmental bacteria (genomes, regulators, proteins, metabolites …) but we still fail to understand the systemic software that it runs. The **second** conclusion is the clear shift from the geno-centric Environmental Microbiology that has dominated the field for a long time to a proteo-centric and chemo-centric vision of the same subject in natural setups. There seems to be a growing perception that just doing more (meta)genomes and (meta)transcriptomes may not produce the scientific dividends that were expected and that we badly need to understand the (bio)chemical logic of microbial communities. Perhaps we need to change the *selfish-gene* paradigm that has so much influenced our approaches to Microbial Ecology by a *selfish-metabolism* counterpart. In this case, the driving force for microbial dynamics is not so much the survival and spread of DNA, but the thermodynamically feasible conquest of the available chemical space. This is, in any case, quite a challenge, because –unlike proteins, metabolism cannot be deduced rigorously from the genome. Fortunately, spectacular advances in metabolomics are being made at the time of writing this article. **Third,** the stunning progress of single-cell methods, from cell cytometry to *in situ* ultra high resolution chemical imaging with a Secondary Ion Mass Spectrometer

(SIMS) allows a new understanding of microbes-in-the environment as the result of highly diverse individual behaviour in otherwise genetically identical populations. This lays the basis for a well grounded *microbial sociology* where division of labour, phenotypic variations and communal co-operation / competition may help to understand and predict biological functions.

In sum, the Baeza Workshop showed us how far we are still from understanding the microbes that rule our planet –let alone to program them for doing what we want them to do. But in the meantime we have moved the frontier of knowledge much further than it was in the late 80s, when the field was somehow established. One of Ken Timmis' favourite remarks is that the success of a scientific meeting is to be measured by the number of experiments that attendees figure out to do as soon as they are back to their respective laboratories. If that is so, we are sure that this workshop was extraordinarily efficacious in inspiring participants (and beyond) to undertake new and exciting lines of action.

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