

Antibiotics as signalling molecules

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We present the argument that the majority of low-molecular-weight organic compounds made and secreted by microbes play roles as cell-signalling molecules in the environment. Of the large number of compounds isolated to date, only a small fraction have been shown to possess useful therapeutic antibiotic activity. However, most microbial metabolites modulate gene transcription at low concentrations, and this is proposed to be the primary effect of the compounds in the maintenance of microbial communities in the environment. Thus, microbial metabolites constitute a large collection of cell-signalling molecules that regulate gene expression in microbial populations and possibly the interactions of these populations with the surrounding organisms.

Keywords: transcription modulation; microbial communities; small molecules

1. INTRODUCTION

The number of microbial taxa in the biosphere is enormous and their distribution is ubiquitous. E. O. Wilson once stated that ‘microbial diversity is beyond practical calculation’; nonetheless, estimates based on experimental observations have been made (Whitman *et al.* 1998) and some current figures are shown in table 1. It should be noted that such estimates of the number of environmental prokaryotic taxa have recently jumped 100-fold as a result of more sophisticated calculations (Gans *et al.* 2005); the numbers certainly represent an upwardly mobile target! The point is that any given environment has a huge, mixed population of prokaryotes (except perhaps under the most extreme conditions, such as the presence of high concentrations of heavy metals). It is known that bacteria do not live isolated lives in nature; they exist in communities. While the production of compounds with antibiotic activity is consistent with the common perception that such diverse communities are highly competitive, we argue that microbial communities are also highly communicative. The two notions are not incompatible. The primary mechanism for controlling all cell functions is regulation of transcription. It has been clearly demonstrated that at subinhibitory concentrations, any antibiotic causes up- or down-expression of a large number of transcripts in different bacteria, many of which determine environmental interactions (Goh *et al.* 2002). But because antibiotic use of small molecules for therapeutic purposes is so strongly entrenched in our experience, the possibility that the so-called antibiotics have other roles as signalling molecules in nature under natural conditions has been ignored.

2. BIOACTIVE SMALL MOLECULES

Whether competitors or communicators, effector small molecules are involved in cell–cell interactions. Based on

microbial numbers in the millions, there must be an exponentially larger number of bioactive compounds produced naturally, probably by most types of living organisms; only a fraction of them have been identified to have antibiotic activity in the laboratory. An antibiotic can be functionally described as a small molecule identified by a pharmaceutical company as having useful therapeutic activity in killing or inhibiting microbial growth. Many small molecules have been isolated and/or used for other therapeutic purposes, as anticancer or antiparasitic agents, or for animal or plant growth promotion (e.g. Demain 2000). The genetic and structural diversity of small molecules produced by microbes showing a variety of bioactivities other than antimicrobial is demonstrated by examining the streptomycetes, a group of bacteria that produce the majority of the natural antibiotics in therapeutic use. The nucleic acid sequence of the genome of *Streptomyces coelicolor*, a representative streptomycete, was achieved in 2002 (Bentley *et al.* 2002). Analysis of the genome sequence indicated the presence of 21 large biosynthetic clusters, each encoding proteins required for the synthesis of a specific small molecule or a group of chemically related small molecules. Three *S. coelicolor* clusters, including the well-studied polyketide antibiotic actinorhodin, had previously been identified to produce molecules having antibacterial properties. Other predicted or known products of the encoded clusters include siderophores, pigments and lipids, as well as uncharacterized molecules. Given the large fraction of the genome dedicated to gene regulation (12.3% or 965 putative proteins), it is reasonable to assume that some of the uncharacterized biosynthetic clusters may encode compounds with undetected signalling roles. In a general sense, not much is known about the biology of small molecules; their biosynthesis is complex and the regulation of their production in the cell has been studied in detail in only a few cases. Furthermore, next to nothing is known of their evolutionary biology and ecology.

Winzer *et al.* (2002) have defined a cell–cell signalling molecule as having the following characteristics: the

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One contribution of 12 to a Theme Issue ‘Bacterial conversations: talking, listening and eavesdropping’.

Table 1. Amounts and cultivability of micro-organisms in nature.

environment	microscopic counts	cultivability
soil	10^{11} – 10^{13} /kg	0.01–0.1%
lakes, rivers	10^9 – 10^{10} /l	0.01–0.1%
ground water	10^7 – 10^8 /l	<1%
marine (surface)	10^7 – 10^9 /l	0.001–0.1%
marine (depth)	10^7 – 10^8 /l	ND
sediments	10^9 – 10^{12} /kg	<1%
small intestine (human)	10^5 – 10^9 /g	10–40%
saliva (human)	10^8 /ml	10–40%

molecule elicits a cellular response which extends beyond the physiological changes required to metabolize or detoxify the molecule; the molecule is accumulated extracellularly and is recognized by a specific receptor; its production occurs during specific stages of growth, under certain physiological conditions and its accumulation generates a concerted response once a critical threshold concentration has been reached. Recently, another view of bacterial communication focusing on the activity of quorum-sensing agents was presented (Keller & Surette 2006). Many antibiotics meet these criteria, perhaps not in the conventional sense, but nonetheless could be described as signalling molecules.

Recent findings have shown that most naturally occurring small molecules have the unexpected ability to modulate global transcription processes in target cells; this activity is detected at much lower concentrations than that required for antibiosis. The characteristic of possessing contrasting effects at low and high concentrations has been referred to as hormesis (figure 1). Studies to date demonstrate that compounds with antibiotic activity have very significant transcriptional activity at subinhibitory concentrations. In bacteria, some 5–10% of all transcripts may be affected (Goh *et al.* 2002); the transcription changes are dependent on the interaction of the small molecule with macromolecular receptors such as the ribosome or RNA polymerase (some examples are listed in table 2). The same macromolecules are the targets of antibiotic activity, but this characteristic may be primarily a laboratory property, since the concentrations of the compounds available in nature would be insufficient to inhibit cell growth. The effect is stoichiometric, and studies to date suggest that transcriptional effects occur at concentrations as low as 1% (or as high as 50%) of those required for inhibition, depending on the compound being used. The pattern of transcription modulation is structure specific and compounds acting at the same receptor may vary. For a review on the effects of subinhibitory concentrations of antibiotics see Davies *et al.* (2006).

Thus, as with conventional signalling molecules, small molecules also have specific cellular targets. While signalling molecules have been identified on the basis of their ability to cause a cellular response, antibiotics have been identified and isolated for their antibacterial properties. Both, as indicated above, can elicit changes that extend beyond any physiological response which may be required to destroy the cell. The number of cellular targets for small molecules is enormous; the

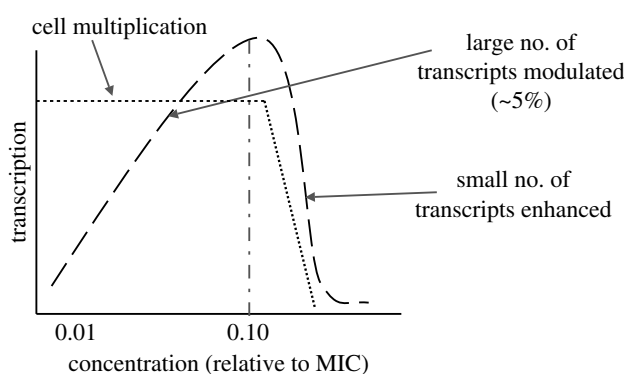


Figure 1. The nature and extent of transcription responses to bioactive small molecules are dependent on concentration. The low concentration effects are considered to be environmentally relevant.

targets are not exclusively receptor proteins on the cell surface but include components of cytoplasmic macromolecular structures. For instance, in the ribosome, any one of the 50 or so ribosomal proteins or ribosomal RNAs has the potential to be a specific receptor to bind small molecules such as the aminoglycoside or macrolide antibiotics. Relatively few of such interactions have been described; studies to date have focused mainly on mutations in ribosomal proteins conferring antibacterial resistance (Poehlsgaard & Douthwaite 2005). The binding of small molecules to bacterial ribosomes has been shown to have hormetic characteristics: transcription modulation or protein synthesis inhibition is dependent on the concentration of the active compound. Binding of small molecules to other macromolecular or enzyme complexes within the bacterial cell wall matrix also promotes dual responses. It has been recognized for some time that small molecules at inhibitory concentrations appear to target primarily key biosynthetic processes.

3. ANTIBIOTICS AND SIGNALLING

Naturally occurring antibiotics (as opposed to synthetic ones) are a subgroup of small molecules that affect transcription of many cellular functions at subinhibitory concentrations (sub-MIC). Synthetic antimicrobials such as the fluoroquinolones or trimethoprim also demonstrate concentration-dependent transcription modulation properties (Li *et al.* 2005; Goerke *et al.* 2006); their action on specific intracellular targets is similar to that of microbial secondary metabolites. This specificity suggests that natural analogues may exist.

Numerous examples of phenotypes associated with antibiotic-induced transcription modulation in bacteria have been reported. The sub-MICs of protein synthesis inhibitors have been shown to both increase and decrease exoprotein production, including virulence functions, in group A streptococci (Tanaka *et al.* 2005). The sub-MIC of cerulenin, the fatty acid synthesis inhibitor, blocks exoprotein production by interfering with specific transcript levels (Adhikari & Novick 2005). Virulence functions can be upregulated; sub-MICs of aminoglycosides, a family of protein synthesis inhibitors, also induce biofilm formation (Hoffman *et al.* 2005). Up and down regulation of the transcription of virulence and motility genes is induced by the

Table 2. Some players involved in antibiotic signalling.

synthetic process	functional receptor		small molecule ligand
translation	ribosome	30S subunit	streptomycin, gentamicin spectinomycin tetracycline
		50S subunit	chloramphenicol erythromycin virginiamycin lincomycin kirromycin mupirocin
transcription	ribosome-bound factors ribosome-associated factors	elongation factor Tu isoleucyl-tRNA synthetase	rifampicin streptolydigin microcin J25
	RNA polymerase	β subunit β and β' subunit β' subunit	
replication	DNA gyrase	subunit A subunit B	novobiocin microcin B17
cell wall synthesis	peptidoglycan synthesis	penicillin-binding proteins MurA LPS alanine racemase and D-alanyl-D-alanine ligase	β -lactam fosfomicin polymyxin D-cycloserine

RNA polymerase inhibitor, rifampicin (Yim *et al.* 2006). Horizontal gene transfer is pandemic in microbial environments and is known to be enhanced by sub-MIC concentrations of tetracycline (Celli & Trieu-Cuot 1998). However, the extent to which gene transfer is dependent on small-molecule signalling is not known. Other activities may include effects on eukaryotes; β -lactam antibiotics stimulate transcription of a glutamate transporter gene in animals (Rothstein *et al.* 2005). If such small molecules produced by defined biosynthetic pathways (and not simply as by-products of primary metabolism) elicit responses in other micro-organisms, can this not be described as signalling?

4. QUORUM SENSING

When one compares the activities of well-known prokaryote signalling agents, such as quorum-sensing autoinducers, with those of bacterial antibiotic-like molecules, there are many similarities. For one, they are produced at defined phases of growth, which is related to nutrient starvation or to some other metabolic change. Structural parallels also exist between known intracellular signals and antibiotics. *Pseudomonas* quinolone signal, a well-characterized intracellular signal involved in the quorum-sensing cascade that regulates virulence in *Pseudomonas aeruginosa* (Wade *et al.* 2005), is a 4-quinolone with structural similarity to the antibacterial 4-hydroxyquinolones ('pyo' compounds) produced by *Pseudomonas* (Calfee *et al.* 2001). Furthermore, autoinducers have been shown to have antibiotic activity. Kaufmann *et al.* (2005) have shown that *N*-(3-oxododecanoyl) homoserine lactone, a product of LasI in *P. aeruginosa*, and its tetramic acid degradation product have antibacterial properties against Gram-positive bacteria. Others have shown that *N*-(3-oxododecanoyl) homoserine lactone and other 3-oxo series homoserine lactones with 8, 10 and 14 length carbon chains inhibited growth of the

Gram-positive *Staphylococcus aureus* (Qazi *et al.* 2006). DNA microarray studies of autoinducers show that they are capable of inducing extensive transcription modulation. Although they are usually described as genus specific in activity (e.g. acyl homoserine lactones and peptides; see Bamard *et al.* 2007; Bjarnsholt & Givskov 2007; Diggle *et al.* 2007; Dong *et al.* 2007; Joint *et al.* 2007; Sanchez-Contreras *et al.* 2007; Williams *et al.* 2007), they may influence the properties of a broad spectrum of target organisms (for example, AI-2). Bauer and co-workers showed that acyl homoserine lactones produced by symbiotic bacteria affect the protein expression pattern of the legume, *Medicago truncatula*, and also that quorum-sensing systems may be important for bacterial-induced nodulation of the legume root, thus demonstrating the importance of small molecules in the communication between plants and bacteria (Mathesius *et al.* 2003; Gao *et al.* 2005).

Many different organisms produce cationic peptides, often referred to as antimicrobial peptides. Although these compounds have been named for their antimicrobial properties, they have been shown to be important activators of innate immunity in humans and animals (Hancock & Diamond 2000). Cationic peptides appear to be non-specific in their activity, affecting both bacteria and eukaryotic cells, as measured by their effects on transcription in the target cells. For example, cecropin, an insect-produced cationic peptide, has been shown to cause significant changes in the levels of 26 transcripts when added to cultures of *Escherichia coli* (Hong *et al.* 2003). The microbial- and mammalian-produced cationic peptides (polymyxins B and E, cattle indolicidin and human LL-37) have all been shown to activate bacterial signalling pathways (McPhee *et al.* 2003). The conclusion is that this plethora of small molecules may signal changes in transcriptional patterns and subsequently the phenotypes of cells which are components of the microbial communities in nature;

the chemical biology/ecology of these compounds deserves more attention.

Interestingly, Selman Waksman, discoverer of streptomycin, the first useful antibiotic produced by a bacterium, observed that ‘antibiotics play no real part in modifying or influencing living processes that occur in nature’ (Waksman 1961). We agree with Waksman’s statement with respect to the inhibitory activity of small molecules, but must point out that the compounds may play important roles in living processes. As mentioned previously, it has been shown that the microbial communities in nature are complex and comprise an indeterminate number of different taxa; in addition, the population is in flux and dependent on the changes in the environment and available nutrients. No studies of signalling have been performed in such intricate environments, but in laboratory experiments, it has been extensively demonstrated that small-molecule signals can affect many cellular characteristics, such as microbial morphology, compound production, pigmentation, etc. We presume that communities are relatively stable and that they are not constantly engaged in microbial genocide. It is more probable that networks of chemical signalling operate to maintain the metabolic stability of the communities in many ways. An understanding of these processes is essential to the study of microbial ecology and may lead to increased discovery of pharmaceutically useful small molecules and better use of the microbial communities for bioremediation and other processes. Microbial behaviour in the laboratory is, at best, indicative of natural roles and cannot predictably reproduce what occurs in any of a variety of natural environments. The relationship of laboratory studies to nature remains unclear; until such time as intercellular and prokaryote–eukaryote (and archaeal!) interactions can be studied *in situ*, they will remain a mystery. Advances in the field will lead to important functional information applicable to the plant and the animal diseases.

5. MICROCINS AND OTHER BACTERIAL SMALL MOLECULES

The bacteriocins (microcins) represent a little studied aspect of small molecule biology. They have not, for some reason, been considered to fit the definition of antibiotics; the argument is specious. These microbial products have been known for some time; they come in many chemical forms and are widely distributed in the microbial kingdom. Laboratory studies have shown that bacteriocins are potent inhibitors of the growth of many bacterial strains, and there is considerable interest in their development as therapeutic agents. The use of these compounds in food protection is commonly practised (for a review, see Cotter *et al.* 2005). Several microcins are produced by enteric bacteria and are believed to be important in controlling the bacterial population of the gastrointestinal (GI) tract through antibiosis (Riley & Gordon 1999); it is unclear how this operates in terms of gut physiology. The microcins have a range of different antibacterial activities and biochemical mechanisms of action; many are derived from small peptides, produced by the normal process of ribosomal protein synthesis, that

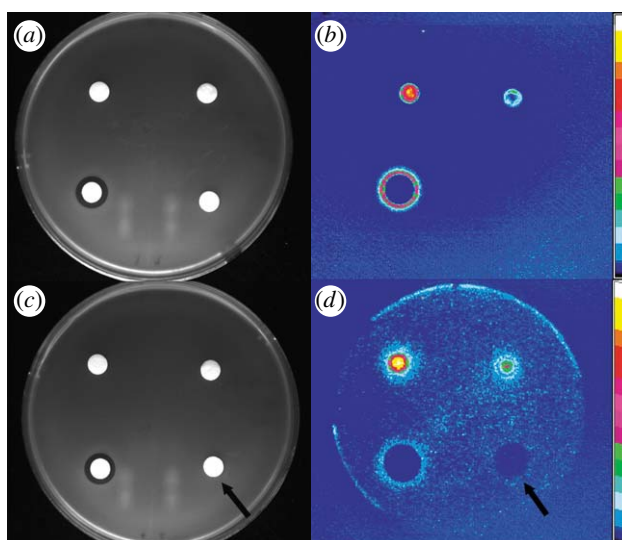


Figure 2. Promoter expression induced by small molecules. LB agar plates with soft agar (0.7% agar) overlays containing 1 : 60 diluted overnight cultures of *Salmonella typhimurium lux* reporter plasmids, where the *luxABCDE* operon is fused to (a, b) the *fadB* and (c, d) the *tsr* genes, respectively. Filter discs containing the following compounds have been placed on top of the overlay: microcin J25, indolicidin, pleurocidin and CP26 (starting upper left and continuing clockwise). Figures (b, d) show light produced from reporter strains converted to the colour scale shown on the far right. The presence of a dark circle around the disc in two dimension containing pleurocidin (see arrow) indicates downregulation of transcription by the compound, as there is no visible growth inhibition in figure (c). The *fadB* and the *tsr* promoters were selected for their general response to various antibiotics and are two promoters used in antibiotic identification panels.

undergo extensive post-translational modification. Microcins are encoded by some of the smallest bacterial genes identified (Gonzalez-Pastor *et al.* 1994). As with the ‘traditional’ antibiotics, microcins have demonstrated antimicrobial activity in the laboratory and accordingly are assumed to be weapons for bacterial warfare in the GI tract. However, the microcins exhibit strong hormesis and show potent transcription modulation with distinct patterns when tested at subinhibitory concentrations (figure 2). We suggest that the ability to induce transient metabolic shifts in gut communities may be a more important role for microcins in their natural environments; they too are signalling molecules. It is probable that the microcins also affect the host cells and the notion should be studied in more detail.

6. RESISTANCE MECHANISMS

In discussing antibiotic activity, one must also consider the question of antibiotic resistance; this serious problem is widely restricting antibiotic use in the treatment of infectious diseases. Interestingly, putative antibiotic resistance genes are common in nature, and many of the resistance mechanisms identified are biochemically similar (and perhaps genetically related) to those found in human and animal pathogens (D’Costa *et al.* 2006). What might be the ecological role of antibiotic resistance mechanisms? Are they essential to protect against antibiosis or do they play roles in cell–cell interactions? We propose that

resistance may serve as a mechanism to modulate the signalling activity of small molecules in nature; endogenous resistance leads to attenuated bacterial strains with specific chemical signals. In recent studies, we have shown that antibiotic-resistant mutants, although they show an altered transcription modulation response to the cognate antibiotic, may acquire a characteristic altered transcriptional response that endows the resistant strain with a phenotype distinct from the parent, sensitive strain in the absence of the antibiotic (H. H. Wang 2006, unpublished data). This suggests that spontaneous mutation to antibiotic resistance in environmental microbes may generate distinct bacterial ecotypes.

7. PROSPECTS AND CHALLENGES

The natural roles of small molecules in biology have been inadequately investigated; it could be argued that this is a field of research in its own right. While their metabolic diversity is not fundamental in a genetic sense, as are DNA, RNA and protein, small molecules are a huge family of biological effectors that influence cellular responses under all conditions; they have specific interactions with many types of macromolecular receptors (Schreiber 2005). We must assume that they are vital to microbial community structure and interactions in the environment. The roles of hormones in human and animal biology are well recognized; are they the evolutionary endpoint of small-molecule signalling in microbes? Anthropocentric approaches have so far provided therapeutic agents that permit the control of human infectious disease, and it has been estimated that drugs derived from microbial metabolites have doubled the human lifespan since their introduction (Verdine 1996). It is evident that a more focused genetic, biochemical and ecological analysis (systems chemical biology?) of the roles of small molecules in cell regulation and communication is likely to provide many other benefits to health and medicine.

Microbiologists are faced with a Herculean task. How does one decipher a language when it is not known who the correspondents are? How does one identify the correspondents in complex mixtures of microbes when more than 99% of them cannot be grown? Significant advances in cataloguing microbial populations have been made in the past 20 years through the application of DNA-based techniques such as polymerase chain reaction; these approaches will continue to be important, but they do not provide information about the behaviour of cells or the language that they speak. A good analogy might be imagined as follows: destroy all of the telephone books and remove all telephone numbers in a city such as New York and try to re-establish the same communication networks from scratch. Everyone has an unconnected and unknown mobile phone! We suspect that solving this metropolitan problem would, in fact, be simple compared with deciphering the logic of inter-microbial communication.

Modern imaging technology permits the visual observation of interactions between small numbers of cells; this technology needs to be improved to allow the scanning of interactions between complex mixtures of cells in soils and other environments. Will it be possible

to superimpose small-molecule signals on these images? Only time will tell.

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