ARE MANY MICROBIAL SECONDARY METABOLITES REALLY PHEROMONES WITH IMPORTANT PHYSIOLOGICAL FUNCTIONS, A KNOWLEDGE OF WHICH COULD GUIDE DISCOVERY PROGRAMMES? DORMANCY, DIFFERENTIATION, SOCIAL RESUSCITATION AND PHEROMONE PRODUCTION IN NONSPORULATING BACTERIA.

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SUMMARY

The functions of secondary metabolites in bacteria are generally not known, although it is to be assumed (and we give evidence) that their production in nature must be of some benefit to the producer organism. Increasing evidence suggests that microbial cultures are differentiated, that intercellular signalling and communication even in prokaryotes is both widespread and (by definition) is effected via pheromone production.We thus develop and review the idea that most microbial secondary metabolites are perhaps best viewed as pheromones, that their production thus represents a form of microbial social behaviour, and that understanding the conditions in which pheromone production is likely to prove important may help to guide the search for novel bioactive molecules of terrestrial and marine origin.

INTRODUCTION AND BACKGROUND

The term "secondary metabolite" was first explicitly applied to microbiology by the late John Bu'lock [1], and notwithstanding a certain arbitrariness [2], Bu'lock's definition distinguished secondary metabolites from the "general" (i.e. primary) metabolites which are produced by most organisms "as having, by contrast, a restricted distribution (which is almost species specific) and no obvious function in general metabolism" [1]. As elsewhere [3], we shall adopt the view that the crucial feature of 'secondary' metabolism is indeed a restricted distribution among a very small number of organisms [4].

Although similar arguments may undoubtedly be applied to other microorganisms such as algae and fungi, and in the present context in particular *marine* microorganisms, our focus will be on the relevant phenomena in bacteria; to this end it is worth pointing out that a number of marine secondary metabolites such as tetrodotoxin that were once ascribed to higher organisms are in fact now known to be the products of symbiotic or commensal

bacteria [5]. Similarly, the recognition of microbial secondary metabolites (which are typically moderately hydrophobic) essentially relies upon their measurement in the extracellular fluid; thus, again virtually by definition, *microbial secondary metabolites are excreted.*

A pheromone is a chemical excreted by an organism into the environment that acts to elicit a specific response from other organisms of the same species. Especially following Stephens' excellent and still little-cited review [6], there is increasing recognition that pheromone production is an important feature of the biology of prokaryotic microorganisms [3; 7-14], and it is thus reasonable to propose that many if not most 'bioactive' secondary metabolites have much of the character of pheromones [3], and that, with some clear but rare exceptions [15-17], the failure to recognise their actions on producer cells is due largely to our inability to perform clearcut experiments on differentiated and often mycelial cultures [4]. A summary of the overall arguments is given in Fig 1.

- The functions of secondary metabolites in bacteria are generally not known.
- It may be assumed that their production in nature must be of some benefit to the producer organism.
- A pheromone is a chemical excreted by an organism into the environment that acts to elicit a specific response from other organisms *of the same species.*
- It is becoming increasingly widely recognised that bacteria can produce pheromones.
- Most axenic cultures are differentiated, and changes require signally
- Most microbial secondary metabolites are thus perhaps best viewed as pheromones
- Hamilton's rule may be used to account for the benefits which such secondary metabolite production afford to the producer
- The recognition of prokaryotic pheromone production opens up a large and novel area of microbial (eco)physiology
- The resuscitation of dormant cells of *Micrococcus luteus* provides an excellent example of microbial pheromone action.

Fig 1. A summary of the views presented here.

PROPERTIES OF PHEROMONES

The general properties of microbial and other pheromones are given in Fig 2.

- In insects, pheromones are involved in signifying the location of food (i.e. the nutritional status of the environment), and in communicating the desire to mate (exchange DNA) and to aggregate
- Very similar roles have been identified for bacterial pheromones, e.g. in iniating sporulation or fruiting body formation, in conjugation, in genetic competence, in swarming and virulence and in the autoinduction of the synthesis of certain 'antibiotics'
- More generally, such hormonal activities may be seen to have a role in effecting developmental change
- Generally active at very low concentrations (< nM - μM)

Fig 2. Overview of the properties and role of pheromones

In microbes, it is occasionally argued that secondary metabolites may be "waste" or "overflow" products [18; 19]; however, a number of simple arguments would suggest that their function must be less gratuitous and more purposeful than that (Fig 3).

- storage product formation is always possible, and the exotic chemical structures and enzymatic pathways would need to have been strongly selected for
- the flux to secondary metabolites in wild-type strains is also rather small to effect much detoxification
- secondary metabolites tend to appear when substrates are *not* in excess [20]!
- uniqueness and structural complexity imply selection
- regulation of their biosynthesis (e.g. under low phosphate conditions) implies a role
- producer tolerance of 'antibiotics' suggests significance to producer
- Genetic organisation: clustering of many 'antibiotic' synthesis genes, together with regulatory genes, implies a highly evolved system that has been strongly selected for

Fig 3. Some arguments why "overflow" metabolism is not the "reason" for secondary metabolite production

Indeed, Davies [21] makes a persuasive case that antibiotic secondary metabolites must at least once have had a role in the physiology and metabolism of the producer organism, and although there is some *limited* evidence that their major role in improving fitness is by affecting competing species, e.g. by antibiosis [22] ("extrinsic functions", Fig 4), most other commentators take the view [4; 23; 24] that more appropriate 'explanations' for secondary metabolism are based on activities that affect the producer species itself (see e.g. [4; 23-30]) ("intrinsic functions"). What type of functions may be postulated?

- "Extrinsic" role, i.e. affecting *other* organisms, e.g. via antibiosis
- There is in fact little evidence that producers gain any competitive advantage from making 'antibiotics' in nature, since the concentrations made by wild-type strains are normally rather small
- There is argument that penicillinase evolved to combate penicillin antibiosis, though the evolution of e.g. clavulanic acid can be interpreted as benefitting the host simply by ensuring continued signals
- In nutrient-rich enviroments it is anyway arguably best to channel resources into turning substrate to biomass as fast as possible, regardless of (first Law) efficiency or growth yield [31; 32]

Fig 4. Does secondary metabolite production help the producer via an extrinsic role, e.g. in antibiotic activity?

The view that secondary metabolites may be evolutionary relics with no real *present* function, though they had one in the past, has been persuasively argued by Davies [21] and does point to a role within the producer organism *itself*. The basic argument seems to be that the "reason" why antibiotics such as streptomycin bind e.g. to the protein-synthesising apparatus is that they were once necessary cofactors for it, and while the subsequent development of proteinaceous machines has taken over this role the one-time cofactors have not been eliminated and can still bind to their receptor sites. However, whilst this neatly accounts for their antibiotic activity at higher levels, it does not seem to us to give a completely obvious explanation for their selective production by a normally very restricted number of species.

As to an "intrinsic" role, i.e. one which brings benefit to the producer strains themselves, one can immediately single out siderophores [33; 34] and mineral scavengers, which are secreted and taken up, where individual molecules tend to have something of a restricted species distribution, and which give a clear benefit to the producer organism(s). However, these siderophores perhaps constitute a slightly special case, and are not entirely within our present compass.

It is certainly also true that morphological changes often accompany differentiation induced by pheromones [4; 7; 8; 28], but that the problem of showing a genuine role *in vivo*, and especially in submerged batch culture where differentiation is suppressed [4], is rarely overcome successfully. It is also worth mentioning that in one sense pheromone function is not strictly 'intrinsic' since the benefit is not (only) to the producer *organism* but to other closely related individuals [3].

If we consider an action by an individual organism (such as the production of a pheromone), it has a cost in terms of a decrease in the number of its own offspring denoted c and a benefit in the increase of the recipient's offspring denoted b. The donor and the

recipient are related to each other by a degree of relatedness r [35]. Hamilton's rule [36-38] then states that the social action is then favoured by selection if rb - c >0. For microbes in a colony in nature, "the cell is the organism", r is essentially equal to 1, and Hamilton's rule thus gives a quantitative measure of *why* pheromone production is likely to be beneficial even to the producer cell [3].

BACTERIAL COOPERATION: THE BREAKING OF DORMANCY AND DECREASE OF LAG *VIA* SOCIAL INTERACTIONS IN *MICROCOCCUS LUTEUS*

In a series of recent studies of the copiotrophic soil bacterium *Micrococcus luteus* [3; 39-47], we have demonstrated (i) that viable and non-viable are not the only 2 physiological macrostates open to nonsporulating bacteria [42] (Fig 5), (ii) that such cells can enter a dormant state from which (by definition) they may be resuscitated [40; 41], and (iii) that resuscitation is a social phenomenon: viable cells can secrete a factor required for the resucitation of their comrades [40; 41; 47]. This makes excellent sense as per Hamilton's rule (Fig 6). The point may be elaborated as follows.

Starved organisms are in a quandary; they cannot stay awake waiting for nutrients which may come, since this requires energy and they are short of it. Neither can they all sleep, since the arrival of new nutrients would then go unnoticed. One excellent solution is thus to differentiate, and have *some* of their number act as 'sentinels' or lookouts [48] that can signal the arrival of new nutrients by 'waking up' their genetic kin, most rapidly via an autoinduction mechanism in which awaking cells themselves are induced to synthesise the wake-up molecule. In the *M. luteus* case, as with pheromone systems from most other Gram-positive organisms (see e.g. [3; 13; 49-58]), the factor is proteinaceous. In addition, this factor can serve as a growth factor or hormone for these cells [59], a phenomenon that we consider will be of increasing significance.

Physiological State	**Phenotype**
Viable	Capable of division; will form a colony on an agar plate.
Vital or dormant	Unable to divide or to form a colony on an agar plate without a preceding resuscitation phase
Non-viable	Incapable of division; will not form a colony on an agar plate under any tested condition

Fig 5. The major physiological states of nonsporulating bacteria [42]. Note that we equate viable with culturable, so we consider the so-called 'viable-but-not-culturable' bacteria [60] to be more properly referred to as 'metabolically-active-but-non-culturable' [61].

- Bacteria in nature experience a f(e)ast and famine existence [62; 63]
- Starved nonsporulating bacteria can enter a dormant state in which they metabolise only very slowly, and cannot form a colony
- When nutrients arrive, cells 'wake up', and excrete a substance which wakes up their friends
- This makes sense, since the cells wish to maximise the rate at which the nutrients are metabolised to their own biomass (i) *per se* and (ii) to beat their competitors
- If r = 1, the benefit will clearly exceed the cost
- Pheromones are thus the key to rapid response in a changing world

Fig 6. Dormancy and social resuscitation.

SOME COROLLARIES OF THE VIEW THAT MANY MICROBIAL SECONDARY METABOLITES ARE PHEROMONES

The benefits of culture differentiation, referred to by Koch as "Insurance policies", have been cogently argued [64], and imply both heterogeneity, even in simple laboratory cultures of *E. coli*, and *ergo* developmental signals. That typical prokaryotic cultures *do* differentiate seems certain, and for a clear and quantitative example of culture differentiation in *M. luteus* see e.g. [39; 40], where rhodamine 123 uptake by individual cells of the organism varied over more than 3 orders of magnitude in the nominal steady state of a slowly growing ($D = 0.01\ h^{-1}$) chemostat culture of the organism. Under these circumstances, only single-cell analyses, such as by multiparameter flow cytometry [45; 65-68], will permit an adequate estimation of the extent of such differentiation.

When viewed quantitatively there should be strong pheromonal selectivity and even specificity for organisms that can coexist; where available, the evidence suggests that this is essentially true, and that organisms that share the same pheromone are sufficiently different ecologically that they are unlikely to come into contact (e.g. the terrestrial plant pathogen *Erwinia* and marine *Vibrio* spp.) [3].

The existence of pheromones implies the existence of receptors. There is now abundant evidence for receptors in prokaryotes, not only for the better-characterised low-MW pheromones of prokaryotes but for a variety of hormones from 'higher' organisms [59; 69-71]

If secondary metabolites are really pheromones and send messages between producer organisms, secondary metabolite formation should be characteristic of habitats with rapidly-changing nutrient status and of organisms with obvious developmental changes. A definitive view on the former does not yet seem to be to hand, although the latter is apparently true; actinomycetes and streptomycetes are the most abundant producers of

secondary metabolites [72] and even myxobacteria [73] are now recognised as important sources of bioactive molecules. In the present context, however, it seems clear that the search for bioactive *marine* metabolites could beneficially be aimed at habitats in which rapid environmental and especially nutritional changes are significant. These would presumably include coastal zones and cognate areas with rapid upwellings of nutrients.

According to the 'pheromone' theory of secondary metabolites formation (which is not intended to be a unitary theory - there are clearly other roles), so-called "antibiotics" should have pheromonal effects. This is true in the sense that they at least have effects on the producer strains, and known microbial pheromones could and do [15-17] have antibiotic effects and other biological effects. This is also true in that at least some butanolides similar to A-factor and the *Streptomyces virginiae* butanolides are antibiotics, and it is worth mentioning that indeed many antibiotic and pheromonal substances are lactones. It is certainly the case that rather few lactones appear as *primary* metabolites.

From a biotechnology or fermentation technology point of view, it is reasonable that many commercial fermentation have not adequately recognised the role of autoinducers in the production of the product of interest, and that (given their potency) the controlled addition of these substances might be expected significantly to improve product yields. This is certainly true in at least one recently established case [74].

PROBLEMS AND PROSPECTS

We trust that the present reasoning has served to provide a fresh perspective on that old chestnut of "why" bacteria make secondary metabolites, that we have been able to stress the ecological and evolutionary significance of such activities, and that the accompanying understanding and further considerations (Fig 7) may serve to guide investigators to more fruitful search and discovery propgrammes for the exploitation of microbial biodiversity.

- Extension to yeasts/fungi (well-known mating hormone systems) and even higher unicellular eukaryotes (evidence for autocrine systems in *Tetrahymena* and *Paramecium*) [75-79]
- Evidence for presence of primitive insulin-like and other polypeptide hormonal substances even in *E. coli*
- Ecological relevance of specificity
- Is there a sensible distinction between pheromones and secondary metabolites, or should we just consider them both as parts of a developmental signalling cascade
- Pheromones *vs* hormones
- Further predictions of optimal habitats for seeking bioactive metabolites?
- Pheromones as microbial growth factors/cytokines?

Fig 7. Some further directions for pheromone and secondary metabolite research.

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