Journal of Industrial Microbiology (1996) 17, 424–431

Autocides and a paracide, antibiotic TA, produced by *Myxococcus xanthus*

E Rosenberg[1] and M Dworkin[2]

[1]*Department of Molecular Microbiology and Biotechnology, George S Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Israel 69978;* [2]*Department of Microbiology, University of Minnesota, Minneapolis, Minnesota 55455-0312, USA*

Myxococcus xanthus **produces two categories of low molecular weight antibacterial materials, autocides and paracides, that have diametrically opposite host ranges. Low concentrations of autocides lyse *M. xanthus*, the producing organism, whereas paracides exert their effects on other bacteria. Antibiotic TA (a paracide) kills all growing bacteria tested that have a peptidoglycan cell wall except *M. xanthus*. It is a macrocyclic polyketide with a molecular weight of 623. The two major autocides produced by *M. xanthus* are phosphatidylethanolamine and a mixture of fatty acids. The modes of action, host ranges and biosynthesis of antibiotic TA and the autocides are presented, and then an attempt is made to explain their role in the complex life cycle of *M. xanthus*. In addition, the remarkable adhesion properties of antibiotic TA and a new semisynthetic derivative of it, focusin, are presented.**

Keywords: *M. xanthus*; antibiotic TA; autocide; paracide; autolysis; development; focusin

Introduction

The myxobacteria are microbial omnivores that feed on other microbes, as well as on a variety of insoluble, macromolecular debris [2,5,30,32,48]. They have evolved effective stratagems to optimize this. They produce a potent battery of extracellular hydrolytic enzymes [2,3,51], and they travel in packs so as to increase the localized concentration of these enzymes [10,11,39,46]. In addition they produce a substantial number of antibiotics [37], and it has been proposed that these antibiotics may play a role in incapacitating their microbial prey [33]. This has been supported by findings on the role of antibiotic TA in the predation of *Escherichia coli* by *Myxococcus xanthus*. The myxobacterium most commonly studied is *M. xanthus*. Fruiting bodies of a closely related myxobacterium, *Myxococcus fulvus*, are illustrated in Figure 1.

The complex life cycle and social behavior of the myxobacteria are related to their need to maintain a high cell density population—a wolf pack—which, by means of excreted proteases, cell wall lytic enzymes, nucleases, etc can solubilize most living or dead particulate biomaterial. *M. xanthus* excretes three additional groups of inhibitory molecules. These are antibiotics [37], which we have chosen to refer to as 'paracides', autocides [56], which induce autolysis during development; and an ill-defined category of inhibitory molecule(s) that seems to be involved in staking out territory [49].

The life cycle of *M. xanthus* can be thought of as comprising two interlocking lifestyles and is illustrated in Figure 2. In one lifestyle, the bacterium grows vegetatively, dividing by binary transverse fission. The organism can be grown on a complex or defined medium in liquid or solid culture, grows exponentially with a reasonable generation

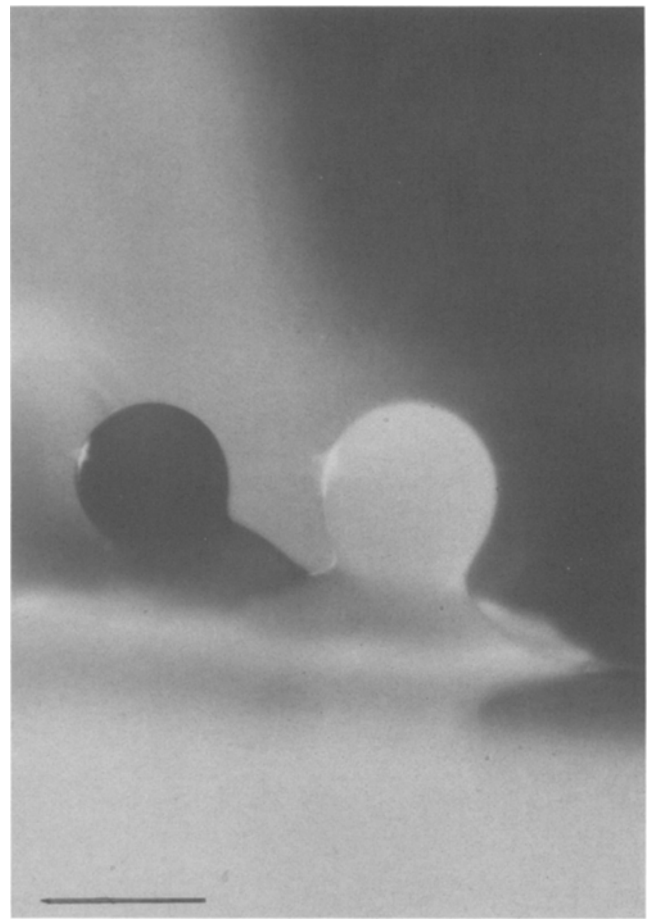

Figure 1 *Myxococcus* fruiting bodies. Phase contrast bar = 50 μm. Courtesy of Hans Reichenbach.

Correspondence: Professor M Dworkin, Microbiology Department, UMHC Box 196, University of Minnesota, Minneapolis, MN 55455-0312, USA
Received 20 February 1996; accepted 28 May 1996

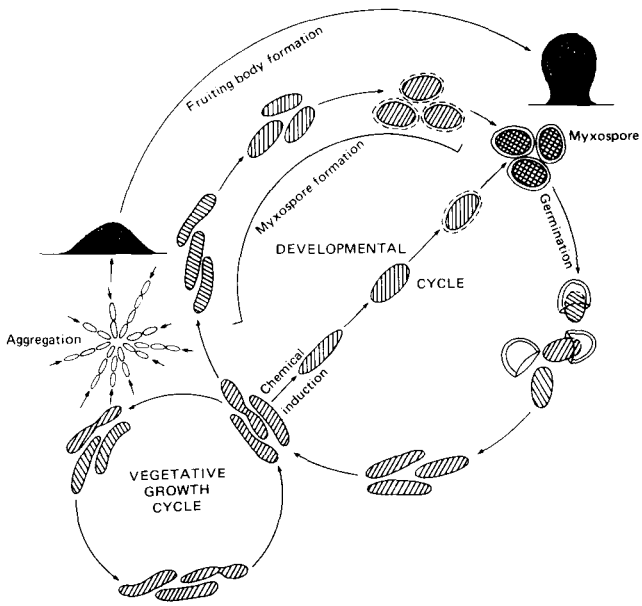

Figure 2 Diagram of the life cycle of *Myxococcus xanthus* [28]. The fruiting body is not drawn to scale but is a few hundredths of a millimeter in diameter. The vegetative cells are about 5–7 × 0.7 μm.

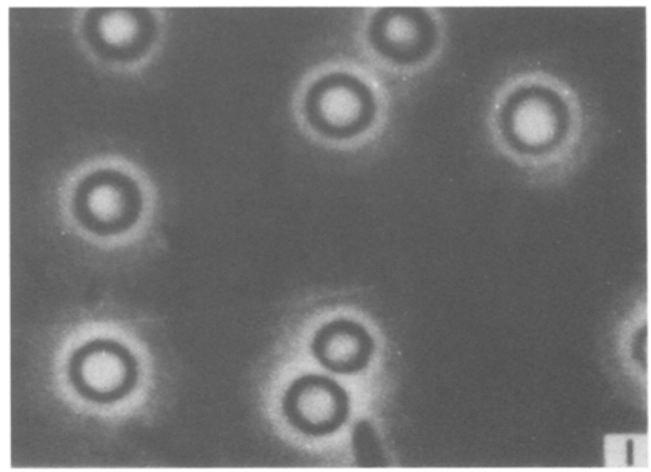

Figure 3 Phase contrast micrograph of myxospores from a fruiting body of *Myxococcus xanthus* [16]. The myxospores are about 1.5 μm in diameter.

time and behaves in most respects as an ordinary, garden-variety Gram-negative prokaryote. Its distinctive nature becomes manifest when *M. xanthus* shifts from its vegetative to its developmental lifestyle. This occurs when three conditions are satisfied. First, the organism must perceive a nutritional shiftdown. It has recently been shown [47] that (p)ppGpp is part of the signal transduction mechanism for responding to the nutritional change. Second, the cells must be on a solid substratum. The myxobacteria move by gliding on a solid surface rather than by swimming. The mechanism of this mode of motility is unknown [4,28]. The gliding motility allows the cells to move into aggregation centers in a fashion superficially analogous to the aggregation of the cellular slime molds. However, unlike its eukaryotic counterpart, *Dictyostelium discoideum*, it does not seem as if the tactic clue is the perception of a gradient of a chemoattractant [14]. Finally, the cells must be present at a sufficiently high cell density. The dependence of *M. xanthus* on the products of hydrolysis of macromolecules generated by its excreted hydrolytic enzymes places the organism at the mercy of diffusion. Its excreted enzymes diffuse away from the cell and the products of the extracellular hydrolysis must diffuse back to the cell as nutrients. Thus, it is critical that the cells feed at a high cell density. And, in fact, it has been shown experimentally that the growth of *M. xanthus* on macromolecular substrates is strictly cell density-dependent [39].

When these three criteria are satisfied, the cells move into the aggregation centers, construct the multicellular fruiting bodies (Figure 1) and then convert to round, optically refractile myxospores (Figure 3). The myxospores are resistant to desiccation, physical disruption and to partially elevated temperatures [50]. In addition they are metabolically quiescent [15], and will remain so until the appropriate nutritional conditions occur and induce their germination [17].

During this complex behavior the cells are interacting with each other [46]. They do so by means of the exchange of chemical signals [22] as well as by contact-mediated interactions [1]. These are generally positive interactions. However, these organisms also compete with each other [49] as well as with other bacteria for territory, they may be feeding on other bacteria [5,48], and they also seem to need to undergo a process vaguely similar to apoptosis in higher organisms [59].

The myxobacteria produce an extraordinary number and variety of antibiotics. This area was recently and comprehensively reviewed by Reichenbach and Höfle [37], and the reader is referred there for a detailed description of chemical structures and modes of action of more than 25 myxobacterial antibiotics. This article will, however, focus its attention on one myxobacterial antibiotic, TA, which has unusual properties and shows considerable promise for clinical use.

The social and developmental biology of the myxobacteria has been reviewed recently [12,13,35].

Properties of antibiotic TA

Antibiotic TA was the first antibiotic isolated from a myxobacterium [41]. Although the antibiotic was purified to homogeneity and crystallized in 1974 [55], its chemical structure was elucidated only by comparison to the subsequently discovered myxovirescin [54]. The chemical structure of myxovirescin A and antibiotic TA is shown in Figure 4. Antibiotic TA and myxovirescin A are isomers; they have identical NMR and UV spectra, but different melting points [36,38]. Furthermore, the two antibiotics differ in host range, the effect of Ca^{2+} ions on activity and the stimulatory effect of serine and alanine on production [19].

Chemically, antibiotic TA is a macrocyclic compound which contains both lactone and amide linkages in the ring. All of the hydrophilic groups, two *cis* vicinal and one primary hydroxyl groups, are both located on one side of the molecule, making it amphipathic and possibly explaining its unique binding properties. The ring can be opened by

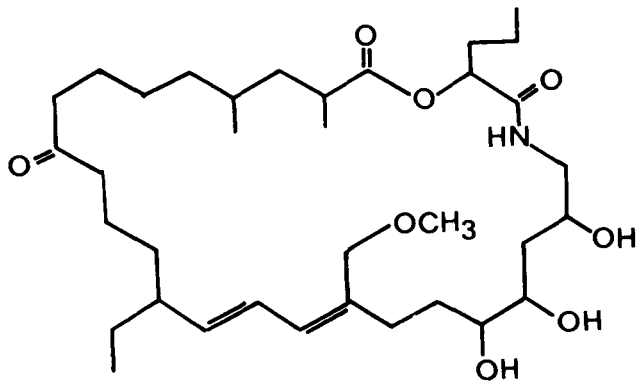

Figure 4 The chemical structure of antibiotic TA and myxovirescin A.

mild alkaline hydrolysis or by periodate oxidation [38]. In both cases, the antibiotic loses its biological activity.

Low concentrations of antibiotic TA inhibit peptidoglycan synthesis without effecting the initial rates of synthesis of DNA, RNA and protein [41]. Spheroplasts can be prepared by adding the antibiotic to growing cultures of *Escherichia coli* in the presence of sucrose and $MgSO_4$. The precise step in peptidoglycan synthesis that is inhibited by antibiotic TA was demonstrated by showing that the antibiotic inhibits incorporation of diaminopimelic acid and uridine diphosphate-*N*-acetylglucosamine into *E. coli* without altering the ratio of cross-linked to noncrossed-linked peptidoglycan [60]. Thus, TA inhibits the polymerization step in cell wall formation, leading to an accumulation of the lipid intermediate. A somewhat similar mode of action has been described for vancomycin [34].

A list of bacteria tested for sensitivity to antibiotic TA is shown in Table 1. The antibiotic has a wide spectrum of activity. Most bacteria were killed by less than 5 μg ml^{-1}. *N. gonorrhoeae, H. pylori* and *A. actinomycetemcomitans* were killed by 10 ng per ml. A small group of bacteria, including two species of *Pseudomonas* and three of *Streptococcus* were killed by only 25 μg per ml of antibiotic TA. The producing organism *M. xanthus*, is resistant to 25 μg per ml. Yeast and other fungi that were tested were not sensitive to the antibiotic.

Table 1 Activity of antibiotic TA against bacteria[a]

Very sensitive (0.01–5 μg ml^{-1})	Sensitive (5–25 μg ml^{-1})
Gram-negative bacteria	
Escherichia coli B	*Serratia marcescens*
E. coli K12	*Pseudomonas fluorescens*
E. coli CW 3747	*Pseudomonas putida*
Klebsiella pneumonia	*Vibrio cholerae*
Proteus morgani	
Neisserria gonorrhoeae	
Actinobacillus actinoymycetemcomitans	
Capnocytophaga sp	
Photobacterium	
Shigella dysenteriae	
Shigella flexneri	
Acinetobacter calcoaceticus	
Helicobacterium pylori	
Gram-positive bacteria	*Streptococcus pyogenes*
Bacillus cereus	*Streptococcus mutans*
Bacillus subtilis	*Staphylococcus aureus*
Bacillus pumilus	*Streptococcus oralis*
Serratia marcescens	
Corynebacterium diphtheriae	
Staphylococcus pyogenes	
Arthrobacter	
Sarcina	
Acintomyces naeslundii	

[a]Taken from Ref. 41, and unpublished data.

Adhesion properties of antibiotic TA to tissues

The most remarkable property of antibiotic TA is that it adheres avidly to a variety of surfaces without losing its activity [40]. This property was discovered while attempting to understand the reason the antibiotic did not protect mice or chicks infected with lethal doses of pathogenic bacteria. Initially it was assumed that the antibiotic was inactivated by blood or tissues. However, incubation of the antibiotic with blood or fresh liver homogenates showed only a slow loss of activity over 24 h.

When antibiotic TA was injected directly into one of the wing veins of a chick, no antibiotic was found in blood samples taken from the other wing for time periods ranging from 1 min to 20 h. After 20 h the chick was dissected and antibiotic extracted from various tissues. About 40% of the input TA activity was recovered from the injected wing. Apparently, the antibiotic diffused from the point of injection only to a limited extent. No antibiotic was found in other tissues, such as heart and lungs.

Oral adminstration of antibiotic TA resulted in the localization of the antibiotic in the crop and grinding stomach of chicks [40]. Two hours after introducing the antibiotic into the crop, 70% of the input activity was recovered in various sites of the digestive tract. The crop (31%), grinding stomach (19%) and oesophagus (10%) contained most of the activity. After 24 h, all of the recovered activity (16% of the input) was found in the grinder contents and membrane. A portion of this membrane, when added to 1 ml of exponentially growing *E. coli*, caused complete lysis of the culture in 2 h. These data suggest that antibiotic TA retains bactericidal activity when bound to membranes.

The observation that antibiotic TA administered to chicks binds avidly to tissues and retains its activity in the bound form led us to examine directly the adhesion, stability and activity of antibiotic TA when applied to mouse tissues [40]. Segments of mouse bladder were treated with various antibiotics *in vitro*, rinsed to remove unbound antibiotic and then added to exponentially growing *E. coli* (Table 2). The only antibiotics effective on the bladder segments following the washing procedure were antibiotic TA and, to a lesser extent, tetracycline. Antibiotic TA also bound avidly to other tissues, including lung, eye, intestine, heart and teeth [27].

Relatively large doses of antibiotic TA were not toxic to rats. Subsequently, the adhesive properties of antibiotic TA were used to successfully treat eight volunteers suffering from severe gingivitis [26]. Four applications of 0.2 mg TA dramatically reduced plaque, gingival and bleeding indices. The effect was retained for at least 30 days after the treatment.

Table 2 Bactericidal activity of antibiotic bound to bladder segments. Antibiotics were applied to freshly-excised segments of mouse bladder, washed twice with saline and then incubated with 1 ml of exponentially-growing *E. coli*. Viable cells per ml were determined initially and after 3 h (incubation 1). Bladder segments were removed after 3 h, rinsed twice in sterile buffer and placed in a tube containing fresh exponentially-growing *E. coli* for an additional 3 h (incubation 2). The procedure was repeated an additional time (incubation 3)

Antibiotic	μg per bladder	Viable cells during					
		Incubation 1		Incubation 2		Incubation 3	
		initial	final	initial	final	initial	final
None	—	1×10^7	3×10^8	8×10^5	2×10^8	1×10^6	2×10^8
TA	16	1×10^7	$<10^2$	8×10^5	1×10^4	1×10^6	6×10^5
Tetracycline	25	5×10^6	3×10^5	8×10^5	2×10^8		
Ampicillin	32	2×10^6	5×10^7	2×10^6	2×10^8		
Cephalosporin C	200	1×10^7	3×10^8				
Naladixic acid	20	1×10^7	3×10^8				
Resprin[a]	50	8×10^6	2×10^8				

[a]Resprin is a 2 : 1 mixture of trimethoprim and sulphamethoxazol.

Molecular genetics of antibiotic TA production

Transposition of TnV and Tn5*lac* into *M. xanthus* yielded 24 TA$^-$ mutants out of 8381 kan^R clones tested [57]. Southern blot analysis of restriction digests of the TA mutant DNA indicated that the transposons were inserted at different sites on the *M. xanthus* chromosome. The TA genes were mapped by cotransduction between pairs of mutants following replacement of the initial insert of one of the pair with the tetracycline-resistant transposon Tn5-132. Most of the TA genes were clustered in a *ca* 40-kb region of the chromosomal P fragment [6]. When Tn5*lac* is transcribed into a transcriptional unit in the correct orientation, it generates a transcriptional fusion to *lacZ*, placing β-galactosidase expression under the control of the promoter for that transcriptional unit [23]. Thus, by measuring β-galactosidase activity in *M. xanthus* strains containing Tn5*lac* in TA genes, it was possible to study the transcriptional regulation of genes required for antibiotic production [53]. The β-galactosidase specific activities were higher when the cells were grown in nutrient-limited medium (139–367 U mg^{-1} of protein than in rich medium (11–48 U mg^{-1} of protein). β-galactosidase activity was further elevated when the cells were grown in the presence of magnesium phosphate, which traps ammonium ions. In general, transcription of the TA genes was low during the exponential phase of growth and high during early stationary phase, typical for genes responsible for the production of secondary metabolites. Detailed restriction mapping of the TA DNA cluster (containing Tn*lac* inserts) indicated that all of the TA genes were transcribed in the same direction (Y Paitan, unpublished data).

Antibiotic TA can be classified as a polyketide antibiotic on the basis of its chemical structure and mode of biosynthesis [57]. Alanine, serine and glycine are the major biosynthetic precursors of antibiotic TA [19]. Both carbon atoms and the nitrogen atom of glycine are incorporated into TA. Alanine and serine are deaminated and converted to acetate before being incorporated into the antibiotic. We postulate that a glycine derivative serves as the starter unit, and then 11 acetate extender units are condensed and processed sequentially in a manner similar to that decribed for the biosynthesis of other polyketides [7]. Formation of the lactone would then lead to ring formation. We are currently searching for precursors of TA by incorporation of labelled glycine into the different Tn5*lac* TA$^-$ mutants.

Tetrahydro-antibiotic TA: Focusin

The first semi-synthetic active derivative of antibiotic TA was prepared by hydrogenation (Figure 5). The product, referred to as focusin, was crystallized and its properties compared to antibiotic TA (Table 3). Although focusin is less active than antibiotic TA to most bacteria tested, it has two important advantages. Firstly, antibiotic TA has a half-life of 5 h when applied to a surface and exposed to room light and air, whereas focusin is stable indefinitely under these conditions. Secondly, focusin adheres significantly better to organic and inorganic surfaces. Toxicity tests are now being carried out on focusin.

Natural role of antibiotic TA

Several lines of evidence suggest that antibiotic TA is one of the weapons used by *M. xanthus* to feed on other bacteria. It does not appear to play a role in development since TA$^-$ strains undergo normal fruiting body and myxospore formation when starved on solid surfaces. Wild type strains produce high levels of antibiotic TA when grown on agar containing living or dead *E. coli*. As mentioned, *M. xanthus* is resistant to antibiotic TA.

When *M. xanthus* was grown in competition with *E. coli* (initial concentration, 7×10^6 cells ml^{-1}), it was found that the cell density required to kill *E. coli* and allow *M. xanthus* to multiply was 3×10^6 ml^{-1}, 1×10^6 ml^{-1} and 1×10^8 ml^{-1}, respectively, for wild type, TA-overproducing and TA$^-$ strains. Addition of antibiotic TA to the medium allowed the TA$^-$ strain to grow when inoculated at 1×10^6 ml^{-1}. Qualitatively similar results were obtained when the three *M. xanthus* strains were grown on lawns of growing *E. coli*. We suggest that antibiotic TA is one of cell density-dependent factors, together with extracellular proteinases [39] that allow *M. xanthus* to derive nutrients from other bacteria.

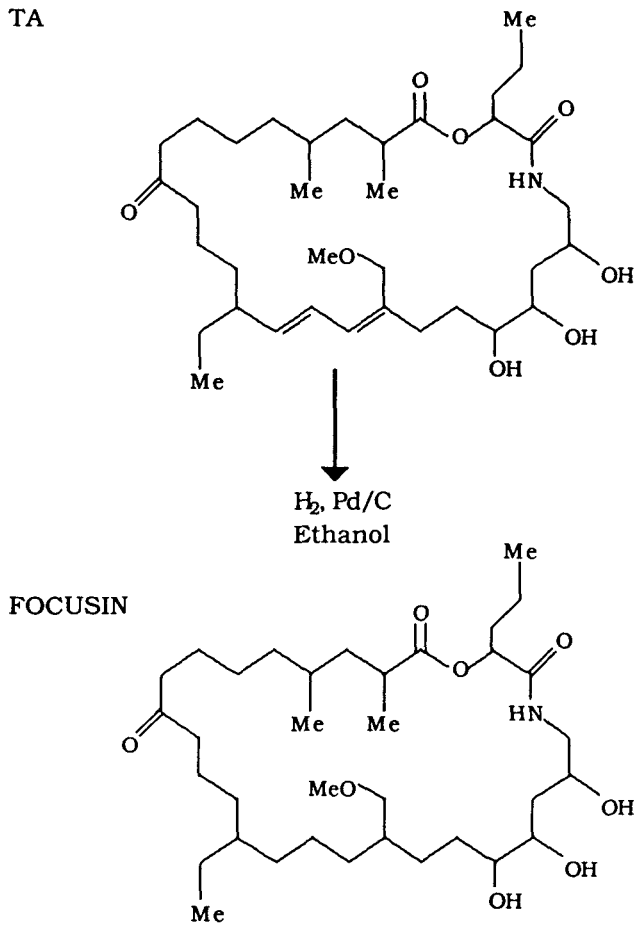

Figure 5 The hydrogenation reaction of TA antibiotic to produce Focusin.

Properties of the *M. xanthus* autocides

Autocides were originally detected as contaminants during the purification of antibiotic TA. The autocide mixture was extracted from *M. xanthus* cells by ethanol and from the extracellular fluid by chloroform [56]. Column chromatography fractionated the mixture into five components, each of which induced lysis in growing or resting cells of the producing and related strains. The two major autocides, termed AMI and AMV, contained 50% and 37% of the total autocidal activity, respectively.

Autocide AMI is composed of a mixture of saturated, monounsaturated and diunsaturated fatty acids [58]. The amount of each fraction required to lyse one milliliter of a *M. xanthus* culture was 10 μg for the saturated, 1.3 μg for the unsaturated and 0.5 μg for the diunsaturated fatty acids. The major fatty acids were the branched *i*-15:0, the monounsaturated *n*-16:1 (*cis*-11) and the diunsaturated *n*-16:2.

Autocide AMV is phosphatidylethanolamine [20], the major lipid in the cell membrane of *M. xanthus* [31]. The autocide activity of AMV is caused by the fatty acid moiety, released by the concerted (enzymatic) activity of many cells. The requirement of high cell density of *M. xanthus* for the lytic activity of AMV is summarized in Table 4. With an initial cell density of 2×10^7 cells per ml, both AMV and AMI killed more than 99.99% of the cells. However, when the initial cell density was lowered to 2×10^6 cells per ml, killing by AMV was reduced to 30% and 0%, respectively. The requirement for high cell density for the killing activity of AMV is abolished in the presence of cell-free supernatant fluids or by addition of phospholipase A_2. In all these experiments, the killing was accompanied by the release of unsaturated fatty acids from AMV. The suggestion that the autocidal activity of AMV is due to the enzymatic release of fatty acids was supported by the demonstration that mutants resistant to AMI were also resistant to AMV [58].

Developmental lysis and myxosporulation

During the first 72 h of fruiting body formation on agar, 80–90% of the starved vegetative *M. xanthus* cells lysed; the remaining 10–20% of the cells became mature myxospores [59]. The fact that lysis and sporulation are interrelated processes was subsequently confirmed in experiments employing microbeads [43]. Sporulation of cells entrapped in 0.6% agarose beads and then suspended in starvation buffer was always accompanied by lysis of the nonsporulating cells. The percentage of lysed cells was correlated with the efficiency of sporulation; eg in an experiment in which 46% of the cells lysed, 3% of the survivors sporulated, whereas when 82% of the cells lysed, 44% of the survivors sporulated. Furthermore, lysis and sporulation were cell density-dependent. If one or two cells in a microbead were starved, they failed to lyse or sporulate. With an average of 4.2 cells per bead, lysis began within 42 h and sporulation within 68 h.

Role of autocides in developmental lysis, sporulation and fruiting body formation

Several lines of evidence indicate that autocides play an important role in the development cycle of *M. xanthus*: (i) mutants resistant to AMI and AMV are defective in fruiting body formation [58]; (ii) low concentrations of AMI (1–3 μg ml^{-1}) accelerate the rate of aggregation and sporulation in wild-type cultures and rescue development in a specific class of signalling mutants (dsg), required for fruiting body formation [42,44,45]; (iii) glucosamine stimulates phospholipase activity and AMI production [29] and also affects development; glucosamine accelerates development when added at low concentrations to wild-type starved cultures, inhibits fruiting body formation at higher concentrations and rescues development in the group C class of developmental signalling mutants [21]; (iv) recently, Downard *et al* [8] described a new class of signalling mutants, designated *esg*. The *esg* locus codes for an enzyme complex that participates in the biosynthesis of branched chain fatty acids [52]. Crude autocide extracts restore development by *esg*-mutant cells [9].

In order to explain the above data, several similar models have been put forth [9,13,20,44]. A composite of these models is shown in Figure 6. During the growth phase, cells synthesize fatty acids, using the product of the *esg* gene to make branched-chain fatty acids. The fatty acids are incorporated into phosphatidylethanolamine (PE), the major lipid of the *M. xanthus* membrane. When the cells become

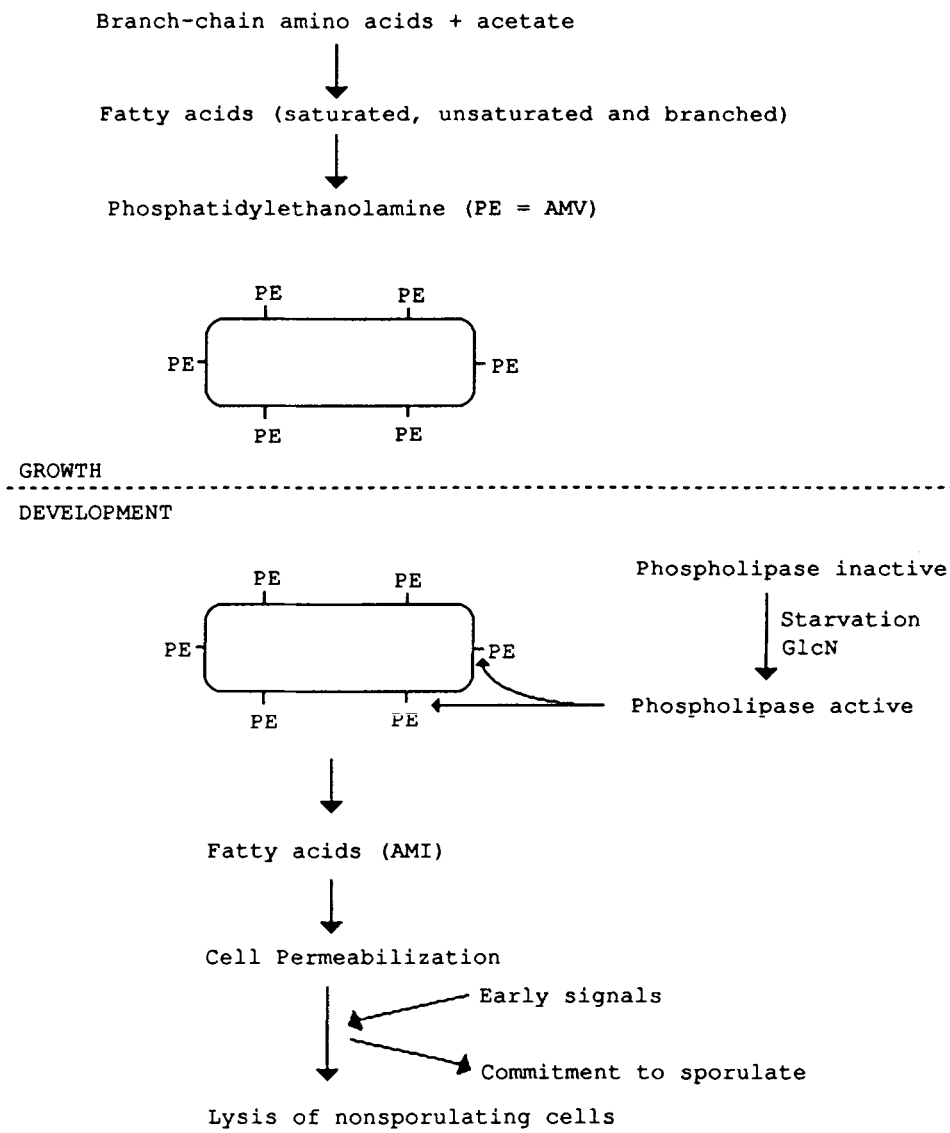

Figure 6 Proposed model for role of autocides in development.

Table 3 Comparison of Focusin to antibiotic TA[a]

Property	TA	Focusin
LD_{50} (*E. coli*)	6 ng ml^{-1}	20 ng ml^{-1}
O_2-light sensitivity	5 h half-life	stable
Polystyrene adhesion (bound/free)[b]	1.0	4.8
Mouse bladder adhesion	0.95 μg	7 μg

[a]Taken from the MSc thesis (1994) of S Alcalay, Tel Aviv University, Ramat Aviv, Israel.
[b]Input: 20 μg ml^{-1}.

Table 4 Cell density-dependent killing by AMV

Inoculum (cells ml^{-1})	Viable cells ml^{-1} with[a]	
	AMV	AMI
2×10^4	2×10^4	$<10^2$
2×10^6	6×10^5	$<10^2$
2×10^7	$<10^2$	$<10^2$

[a]Exponentially growing *M. xanthus* cells were diluted into fresh medium containing 10 μg AMV or 2 μg AMI per ml. Viable cell number was determined after 6 h of incubation. Data taken from Gelvan *et al* [20].

starved, they enter the development stage and phospholipases are activated. The phospholipase can also be activated by addition of glucosamine [29]. The phospholipases hydrolyze the PE, releasing free fatty acids (AMI). The AMI in the membrane causes the cell to become more permeable, allowing for the exchange of extracellular signals. Since cell death is a consequence of addition of autocide AMI later in development (after 13 h), lysis may be a secondary effect of autocides later in development. Increases in the intracellular concentration of glycerol during development in *M. xanthus* have also provided evidence that the glycerol that is released by the hydrolysis of phosphatidyl-

ethanolamine may play a role in myxospore morphogenesis [18].

Finally, it is interesting to compare the mechanism of production and function of autocides in *M. xanthus* with the production and activity of eicosanoids in mammals [24]. In the latter case, phospholipids are hydrolyzed to produce unsaturated fatty acids [25] which are then modified to produce active eicosanoids, including prostaglandins, thromboxane and leukotrienes. In both systems, phospholipids are enzymatically hydrolyzed to produce fatty acids which modify membranes so that signals can be released or taken up by the cells. Whether there was a direct evolutionary connection between the prokaryotic and mammalian biochemical pathways or whether this is an example of convergent evolution is yet to be determined.

Comparisons of autocides with a paracide (antibiotic TA)

It is interesting to compare, in the same organism, two groups of low molecular weight compounds, one which apparently is involved in development (autocides) and the other which is designed to kill other bacteria (ie a paracide, eg antibiotic TA). To begin with, both types of molecules are hydrophobic and interact with bacterial cell envelopes. The autocides make *M. xanthus* permeable to extracellular signals whereas antibiotic TA inhibits the polymerization of the disaccharide-pentapeptide-lipid intermediate. Although acetate is the major precursor of both groups, the autocides are made throughout the growth cycle, whereas the paracide is synthesized, like a typical secondary metabolite, only at the end of the growth phase. The producing strain is insensitive to the paracide, growing and developing normally in its presence. However, the producing strain is extremely sensitive to the concentration of autocide. High concentrations of autocides kill growing or resting cells; low concentrations accelerate development. Mutants resistant to autocides grow normally, but fail to develop. We suggest that a fundamental difference between an autocide (an effector of development) and a paracide (an antibiotic) is that the active concentration of the autocides are highly regulated by modification of a primary metabolite, whereas the paracide is synthesized *de novo* at the end of the growth phase.

Acknowledgements

The work in Eugene Rosenberg's laboratory was supported in part by the Pasha Gol Chair for Applied Microbiology and the Fogarty International Center for Advanced Study in the Health Sciences, NIH. The work in Martin Dworkin's laboratory was supported by National Institutes of Health grant GM 19957 to MD

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