Antimicrobial activity and biosynthesis of indole antibiotics produced by *Xenorhabdus nematophilus*

LATHA SUNDAR and F. N. CHANG*

Department of Biology, Temple University, Philadelphia, PA 19122, USA

(Received 12 February 1993; revised 17 June 1993; accepted 1 July 1993)

We have investigated the mechanism of action and physiology of production of the indole derivative antibiotics produced by the nematode-associated, entomopathogenic bacterium *Xenorhabdus nematophilus*. Maximum antibiotic concentration was reached during the late stationary phase of growth, and the antibiotic yield was appreciably enhanced by supplementation with tryptophan. Antibiotic biosynthesis apparently involved the removal of the side-chain carboxyl (C-1) carbon of tryptophan. The C-3 methylene carbon of tryptophan, on the other hand, was retained. The purified indole antibiotic was effective against both Gram-positive and Gram-negative bacteria at low to moderate concentrations causing a severe inhibition of RNA synthesis, accompanied by a less severe effect on protein synthesis. An isogenic pair of *Escherichia coli* strains differing at the *relA* locus was used to demonstrate that the swift reduction in total RNA synthesis is related to an antibiotic-induced accumulation of the regulatory nucleotide, ppGpp, in susceptible bacteria. The *E. coli relA* mutant, which does not exhibit any discernible increase in ppGpp upon antibiotic treatment, showed no decrease in growth or RNA synthesis. Using this antibiotic, it was also observed that ppGpp may be employed as a metabolic regulator in bacteria such as *Pseudomonas putida*, which have not previously been reported to employ ppGpp as a regulatory molecule. We propose that the indole derivative antibiotic exerts growth inhibitory control in susceptible bacteria by greatly enhancing synthesis of ppGpp, resulting in a rapid inhibition of RNA synthesis.

Introduction

Xenorhabdus spp. belong to a group of diverse and unusual entomopathogenic Gram-negative *Enterobacteriaceae* (Akhurst & Boemare, 1988) which are almost always found in mutualistic symbiosis with two families of insect-parasitic nematodes, the *Steiner-nematidae* and the *Heterorhabditidae* (Poinar & Thomas, 1966; Poinar *et al.*, 1977). In nature, a single species or subspecies of these bacteria are carried monoxenically in the intestinal pouch of the infective stage juvenile nematodes, which are also known as 'dauer' nematodes (Poinar & Thomas, 1966; Thomas & Poinar, 1979). All known species of *Xenorhabdus* have been reported to exist in two phases (I and II). Phase I is normally unstable and easily converts to phase II. The two phases are distinguished by the ability of the phase I (but not phase II) bacteria to produce antimicrobial substances (Akhurst, 1980), to adsorb neutral red from MacConkey agar (Boemare & Akhurst, 1988) and, for most strains,

bromothymol blue from agar media (Akhurst, 1980). Morphologically, phase I colonies are generally mucoid and difficult to disperse in liquid, whereas phase II colonies are non-mucoid and are easily dispersed (Boemare & Akhurst, 1988).

The phase variation plays an important role in the symbiotic relationship with the nematodes. The dauer nematodes, carrying only phase I *Xenorhabdus* in their intestine, invade the insect host through the cuticle (Bedding & Molyneux, 1982) or natural openings like the mouth, anus and spiracles, thus acting as natural vectors for the bacteria (Poinar & Thomas, 1966). Once in the gut, the larval nematodes penetrate into the insect haemolymph, where the released bacteria proliferate rapidly, leading to insect septicaemia and death (Poinar, 1966; Poinar & Thomas, 1966). Furthermore, the developing nematodes may also aid in hastening insect septicaemia by releasing into the haemolymph a toxin (Burman, 1982) which inhibits the insect inducible immune system (Gotz *et al.*, 1981).

Antibiotic production seems to be a crucial feature in this infective process in the insect host. Phase II variants, which generally lack this activity (Akhurst, 1980;

* Author for correspondence. Tel. +1 215 204 8843; fax +1 215 204 6646.

Boemare & Akhurst, 1988), are unable to restrict the invasion of the insect gut by other bacteria which may be detrimental to the nematodes, leading to poor nematode reproduction (Akhurst, 1980; Bedding, 1981, 1984). The antibiotic substances produced by *X. nematophilus* subsp. *nematophilus* have been identified as a group of four closely related indole derivatives (Paul *et al.*, 1981). The antibiotic remains stable under conditions of extreme heat (Akhurst, 1982) and retains its biological activity in the insect cadaver for an extended period of time. This suggests that development of bacterial resistance to the antibiotic may be infrequent.

Not much is currently known about the spectrum of activity of the antibiotic, the effective inhibitory concentrations, the interaction of this antibiotic with susceptible organisms, and the physiology and rate of antibiotic production. In an effort to answer some of these questions, we have studied the mechanism of action and, to some extent, the physiology of biosynthesis of the indole derivative antibiotic produced by *X. nematophilus*.

Methods

Bacterial strains. Antibiotic spectrum assays were conducted using laboratory strains of *Bacillus cereus*, *Micrococcus luteus*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Pseudomonas fragilis*, *Pseudomonas putida*, *Serratia marcescens*, *E. coli* B, and *E. coli* K12 strains JC355 (*metB1 leuB6 hisG1 argG6 relA⁺ spoT⁺ rplK⁺*), NF161 (*argA52 metB1 relA⁺ spoT⁻*) and NF162 (*argA52 metB1 relA⁻ spoT⁻*). Phase I variants of the subspecies *X. nematophilus* subsp. *nematophilus* were obtained from Dr Thomas Schmidt, Indiana University, Indianapolis, USA.

Maintenance and identification of *X. nematophilus* phase I bacteria. The antibiotic-producing phase I *X. nematophilus* were maintained on either nutrient agar (NA) or Luria-Bertani (LB) agar slants and subcultured monthly. Due to the instability of the phase I under normal culture conditions, glycerinated stocks of these bacteria frozen at -70°C were frequently used as starting material for cultures. Phase I was distinguished from phase II by its adsorption of neutral red from MacConkey agar (Difco) and bromothymol blue from NBTA agar (nutrient agar, 0.0025% bromothymol blue, 0.004% triphenyltetrazolium chloride; Akhurst, 1980) media, as well as general colony morphology on these media. On nutrient agar, phase I colonies were generally cream-coloured, with smooth margins, while phase II colonies were translucent, with spreading margins.

Production and purification of indole antibiotics. Seed culture for antibiotic production was performed by inoculating a loopful of phase I *X. nematophilus* from thawed glycerinated stocks, or from single colonies obtained from MacConkey or NBTA agar plates into a 250 ml flask containing 100 ml fresh LB or YSG medium. YSG medium contains (l^{-1}): glycerol, 5 g; yeast extract, 15 g; 1 M- MgSO_4 , 5 ml; $(\text{NH}_4)_2\text{SO}_4$, 2 g; 1 M- KH_2PO_4 , 5 ml; 1 M- K_2HPO_4 , 5 ml; and 1 M- Na_2SO_4 , 10 ml. The LB medium contains (l^{-1}): tryptone 10 g, yeast extract 5 g and NaCl 10 g. In an effort to enhance antibiotic yield by enrichment with precursors, cultures of *X. nematophilus* in the early-stationary phase of growth were supplemented with 1 mM or 1.5 mM concentrations of different amino acids, either individually or as mixtures. Different media such as nutrient broth (Difco), LB, YSG, antibiotic medium (Difco), 2YT (tryptone, 16 g l^{-1} ; yeast extract 8 g l^{-1} ; NaCl, 5 g l^{-1}), YS broth (yeast extract, 5 g l^{-1} ; $(\text{NH}_4)_2\text{SO}_4$,

5 g l^{-1} ; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g l^{-1} ; KH_2PO_4 , 0.5 g l^{-1} ; K_2HPO_4 , 0.5 g l^{-1}) were also used in comparative studies to find the optimal nutrient medium for antibiotic production. All media were adjusted to a final pH of 6.8 to provide optimal conditions of growth for *X. nematophilus*. The bacteria were allowed to grow aerobically at 25°C in a Psycrotherm incubator (New Brunswick Scientific), with the shaker set at 160 r.p.m. Growth was monitored at OD_{720} using a spectrophotometer (Gilford Instruments). At the early-exponential phase of growth, all of the seed culture was transferred into 1 litre of fresh medium in a 2 litre flask and allowed to grow to the stationary phase. The stationary phase cultures were centrifuged for 10 min at 6000 r.p.m. to pellet bacterial cells. The supernatants were combined and extracted three times with equal volumes of ethyl acetate (Paul *et al.*, 1981). The extracts were evaporated and redissolved in small volumes of ethyl acetate. Crude antibiotic extracts were purified on glass-backed silica thin layer plates (20×20 cm; Analtech) by ascending chromatography in a solvent containing n-butanol/ethanol/water (5:1:4, by vol.).

Thin vertical slivers of the dried chromatogram were cut out for use in both antibiotic activity bioassays and colorimetric reactions with Ehrlich's and ninhydrin reagents. The R_F on the chromatogram corresponding to the maximal antibiotic activity on the sliver was noted. The region on the original chromatogram corresponding to this R_F value, as well as the area within 0.5 cm on either side was scraped out and back-extracted in ethyl acetate to recover the antibiotic. The concentration of the purified antibiotic was determined using Ehrlich's reagent, and verified by comparing the zones of inhibition produced by various amounts of this antibiotic against the two standard test organisms, *B. cereus* and *E. coli*.

Physiology and kinetics of antibiotic production. Phase I *X. nematophilus* were grown in nutrient media in a 25°C shaker and 4 ml aliquots of the growing culture were removed at intervals. A sample (1 ml) was used to assess growth by turbidimetric measurement at OD_{720} . The remaining 3 ml of the culture were used for the antibiotic bioassay by extracting with ethyl acetate (Paul *et al.*, 1981). A third of this antibiotic extract was used to determine the concentration by the colorimetric Ehrlich's assay. The remaining extract was used in antibacterial disk diffusion assays (see below) using *B. cereus* as indicator organism. After 16–18 h incubation at 31°C , the plates were checked for antibiotic zones of inhibition.

Radiolabelling of antibiotic with precursors. Phase I *X. nematophilus* were grown in Spizizen minimal medium (Spizizen, 1958) supplemented with all amino acids except tryptophan. *X. nematophilus* grown to the late-exponential or early-stationary phase were labelled with 0.2 μCi (7.4 kBq) ml^{-1} of one of [^3H]tryptophan, [*side-chain-1- ^{14}C*]tryptophan or [*side-chain-3- ^{14}C*]tryptophan. Unlabelled cultures were also maintained for performing antibiotic assays. The cultures were incubated for 24 h at 25°C in a Psycrotherm shaker, at which time the antibiotic was extracted. Crude extracts from the labelled and unlabelled samples were evaporated and redissolved in small, constant volumes by ethyl acetate. A portion of each of these extracts was subjected to ascending chromatography on analytical silica thin layer plates (Analtech) using five different solvent systems: methylene chloride/ethyl acetate (3:7, 2:8, and 11:9, v/v); n-butanol/acetic acid/water (11:5:4, by vol); or hexane/ethyl acetate (11:9, v/v). Chromatography of each extract in a given solvent system was performed in triplicate to ensure reproducibility of results. These solvent systems had been previously selected since they had produced best separation of the different derivatives of the antibiotic extract and modest R_F values for the same.

Detection of radioactive spots on dried chromatograms of the labelled antibiotic was enhanced by fluorography followed by autoradiography. Briefly, the chromatograms were quickly saturated with PPO in diethyl ether (1 g per 20 ml), air-dried for 10 min and exposed to Kodak X-OMAT AR film for 24–36 h at -60°C . The antibiotic spots on the chromatograms were detected by placing the dried

chromatograms of the unlabelled antibiotics on nutrient agar plates which were then overlaid with the indicator bacteria (*B. cereus*) dispersed in soft agar. Zones of inhibition and the R_F values of antibiotic activity were noted after 16–18 h incubation at 37 °C. A second set of chromatograms of the unlabelled extract was used in the colorimetric (Ehrlich's) assay which detects the presence of indoles. The R_F of the antibiotic spot on chromatograms in these assays was compared to the R_F values of radioactive spots from the fluorographed and autoradiographed chromatograms.

Antibacterial action and spectrum of activity. Antimicrobial activity of the purified compounds was assayed by using standardized serial dilution assays and disk diffusion assays. In disk diffusion assays, the efficacy of the antibiotic compounds was compared to that of some commercially available antibiotics. Disks of the indole antibiotics were prepared by incorporating the required concentrations into sterilized 6 mm diam. disks made from Whatman 3 MM paper.

Overnight cultures of the bacteria in Luria-Bertani (LB) nutrient liquid medium were diluted 1:2000 in soft agar and poured over nutrient agar plates to produce bacterial lawns. The air-dried antibiotic disks were placed firmly on the bacterial lawns, along with disks of other commercial antibiotics for comparison of antibacterial activity. The plates were incubated in a Psychrotherm incubator (New Brunswick Scientific) set at either 30 °C or 37 °C, as dictated by the individual growth requirements of the bacteria. Zones of inhibition for the various antibiotic disks were measured as the total diameter of bacterial growth inhibition around the disks.

Standardized serial dilution assays were performed to determine the minimum inhibitory concentrations (MIC) of the antibiotic. MIC was defined as the lowest concentration of antibiotic at which no bacterial growth was observed after 18 h incubation in a rotary shaker (160 r.p.m.) set at the required temperature.

The effect of different dilutions of the antibiotic on eukaryotic cells was studied using cultures of chicken fibroblasts. Cell morphology and density of the cultures before and after addition of the antibiotic were monitored by photography.

Measurement of RNA, DNA and protein synthesis in bacteria. [^3H]Leucine, [^{14}C]uracil and [^3H]thymine were used as precursors for synthesis of protein, RNA and DNA, respectively, in whole cells. The bacteria used in such assays included *B. cereus* and *E. coli* K12. In all cases, 16 h cultures of bacteria in liquid nutrient medium were diluted into fresh Hershey's Tris minimal medium (Chang *et al.*, 1974) supplemented with 0.3% glucose, thiamine (2 $\mu\text{g ml}^{-1}$), and 50 $\mu\text{g ml}^{-1}$ of each required amino acid. The cultures were allowed to reach the exponential phase of growth (approximate OD_{720} 0.45–0.5). Bacteria preincubated for 5 min with 2–8 μg antibiotic ml^{-1} were labelled with 2 μCi [^3H]leucine, 0.4 μCi [^{14}C]uracil or 2 μCi [^3H]thymine ml^{-1} . Aliquots (1 ml) were removed at different times and immediately transferred into 5 ml ice-cold 10% (w/v) trichloroacetic acid (TCA). For the measurement of protein synthesis, the TCA-treated samples were boiled in a 100 °C water bath for 10 min. Samples were passed through 0.45 μm HA Millipore filters, which were then washed three times with 5% ice-cold TCA. The dried filters were placed in 5 ml scintillation fluid and counted in a scintillation counter.

Analysis of ppGpp production by labelling cellular nucleotides with $\text{H}_3^{32}\text{PO}_4$. Bacterial strains were grown at the optimal growth temperature in Hershey's Tris minimal medium containing 0.33 mM-phosphate, 0.3% glucose, 2 μg thiamine ml^{-1} and 50 μg of the required amino acids ml^{-1} . Where necessary, the stringent response in *E. coli* K12 strains was elicited through a valine-induced isoleucine starvation by adding 400 μg valine ml^{-1} (Leavitt & Umbarger, 1962). Cells were labelled with carrier-free $\text{H}_3^{32}\text{PO}_4$ at least one generation before sampling to allow equilibration with the phosphate pools. Time of sampling was arranged to correspond to the mid-exponential phase

(OD_{720} 0.45–0.5) when 800 μl aliquots of cells were transferred to sterile Eppendorf tubes and treated with the appropriate compounds. All samples were incubated for 7 min in a water bath set at the appropriate temperature. While other incubation times were also tried, the 7 min incubation was uniformly used since all the bacteria that we investigated consistently responded within 6–7 min of antibiotic treatment by accumulating substantial amounts of ppGpp. At the end of the incubation period, the tubes were immediately placed on ice to stop enzymic reactions. The tubes were centrifuged in a microcentrifuge for 3 min at 0 °C to pellet the bacterial cells. After discarding the supernatants, cell pellets were subjected to nucleotide extraction by either the conventional formic acid extraction procedure (Cashel, 1969; Lowen, 1976) or the lysozyme–deoxycholate freeze–thaw procedure (Lagosky & Chang, 1978). Following nucleotide extraction, samples were centrifuged at 0 °C in a microcentrifuge for 5 s and the supernatant fraction used for ascending chromatography on pre-washed poly-ethyleneimine (PEI) cellulose thin layer plates. The identities of the nucleotides were verified through comigration of unlabelled nucleotide standards. Radioactively-labelled nucleotides were located by autoradiography, and the corresponding spots were cut out from the thin layer chromatogram. Radioactivity in each spot was determined in a toluene-based scintillation cocktail. Adjustment for the background counts was made by determining the radioactivity in equally sized portions of the thin layer cut out from adjacent non-radioactive areas.

Analysis of cellular nucleotides by thin layer chromatography. PEI cellulose thin layer plates were prewashed in water and dried before use. For one-dimensional chromatography, 3 μl of each sample was spotted in parallel, 1 μl at a time. Ascending chromatography was performed in 1.5 M- KH_2PO_4 at pH 3.4 (Cashel, 1969). In two-dimensional chromatography, 1.5 M-LiCl and 2 M- HCOOH in the first dimension and 1.5 M- KH_2PO_4 (pH 3.4) (Gallant *et al.*, 1976) in the second dimension were used. Immediately following the first dimensional chromatography, the thin layer plates were washed in anhydrous methanol for 20 min to remove excess salts and dehydrate the thin layer, both of which resulted in improved resolution. Depending on the age of the sample, approximately 7–10 μl of the supernatant was used for two dimensional chromatography. For chromatography of lysozyme–deoxycholate extracts, the origin was prespotted with water, followed by spotting of samples to improve resolution and minimize streaking.

Radioactive isotopes and chemicals. Carrier-free orthophosphoric acid $\text{H}_3^{32}\text{PO}_4$, [^{14}C]uracil, [^3H]leucine and [^3H]thymine were purchased from ICN Biochemicals. [^3H]tryptophan, [*side-chain-1- ^{14}C*]tryptophan, [*side-chain-3- ^{14}C*]tryptophan and Formula 949 scintillation fluid were obtained from New England Nuclear. Polyethyleneimine (PEI) TLC sheets (Polygram Cel 300, precoated plastic sheets, 20 $\times$ 20 cm) were purchased from Brinkmann Instruments and from J. T. Baker. Autoradiography was carried out with Kodak X-ray film (X-Omat AR). Commercial antibiotic disks were obtained from local sources. Glass-backed silica TLC plates (20 $\times$ 20 cm) for the purification of the crude indole antibiotic were obtained from Analtech.

Results

Biosynthesis of indole derivative antibiotics by X. nematophilus

The structures of the indole derivative antibiotics are shown in Fig. 1. By monitoring the growth of *X. nematophilus* subsp. *nematophilus* and the production of antibiotics over 4 d, it was found that maximal antibiotic accumulation in the culture medium reached a peak at the late stationary phase (Fig. 2). There was no significant

Fig. 1. Structure of indole derivative antibiotics produced by *X. nematophilus* subsp. *nematophilus* (reproduced from Paul *et al.*, 1981).

Fig. 2. Biosynthesis of indole derivative antibiotics by *X. nematophilus* subsp. *nematophilus*. Phase I colonies of *X. nematophilus* were grown aerobically in LB nutrient medium at 25 °C. Aliquots of the culture were removed at intervals for monitoring growth at OD₇₂₀ and 3 ml samples used for antibiotic extraction. Portions (2 ml) of the extract were used in an antibiotic diffusion assay, with *B. cereus* as the indicator species. Points are the average of three identical studies. ■, Bacterial growth; □, antibiotic production.

increase in antibiotic production beyond this period. The generation time of this bacterium in LB was found to be about 1.5–2 h, in the exponential phase of growth. Addition of tryptophan to the LB medium at a final concentration of 1 mM significantly enhanced the yield of the antibiotic. The production of antibiotics by early-stationary-phase cultures of bacteria grown in different nutritive media was also studied. Bioassays of these extracts indicated that *X. nematophilus* grown in YSG medium produced the maximum yields of antibiotic.

The involvement of tryptophan as a precursor for antibiotic biosynthesis was confirmed by labelling *X. nematophilus* cultures with [³H]tryptophan. Chromat-

ography and bioassay of the antibiotic extracts in three different solvent systems [methylene chloride/ethyl acetate (2:8 and 11:9) or n-butanol/acetic acid/water (11:5:4)] consistently showed that there was substantial incorporation of tryptophan into the antibiotic (Fig. 3). The fate of the carbon side chain of tryptophan during antibiotic biosynthesis was then determined. Fig. 4 shows the comparative incorporation of [*side-chain*-1-¹⁴C]tryptophan and [*side-chain*-3-¹⁴C]tryptophan into the antibiotic as detected by comigration with authentic compounds in different solvent systems. It was found that the incorporation of [*side-chain*-3-¹⁴C]tryptophan into the antibiotic was approximately 15-fold higher than that of [*side-chain*-1-¹⁴C]tryptophan. Since the specific activities of these two radioisotopes are essentially the same [51 and 54 mCi (1887 and 1998 MBq) mmol⁻¹], this would indicate that the side-chain C-1 carbon (carboxyl carbon) is preferentially removed during antibiotic biosynthesis. In all cases, the *R_f* of the radioactive spot on the chromatogram corresponded to that of the antibiotic spot in the antibiotic bioassay.

Antimicrobial activity spectrum

The results of the disk diffusion assays and MIC of the antibiotic are shown in Table 1. Application of 2–6 µg of the antibiotic on the disks inhibited the growth of most bacteria tested. Gram-positive bacteria such as *B. cereus*, *M. luteus* and *B. megaterium* were found to be susceptible to concentrations of antibiotic as low as 1 µg. A pair of *E. coli* K12 strains, NF161 and NF162, differing only at the *relA* locus were also used in this study. Interestingly, the *E. coli* relaxed mutant (NF162) remained resistant to concentrations of the indole derivative antibiotic up to 10 × the MIC of its isogenic, stringently regulated partner, NF161.

The addition of the antibiotic to cultures of chicken fibroblasts appeared to have no deleterious effect on the morphology or growth of the cells, even at concentrations as high as 8 µg antibiotic (ml culture)⁻¹ (data not shown).

Inhibition of the synthesis of RNA and protein

The effect of the indole derivative antibiotics on the incorporation of biosynthetic precursors such as [³H]leucine, [¹⁴C]uracil and [³H]thymine into protein, RNA and DNA, respectively, was studied using *B. cereus* and *E. coli* K12. Fig. 5 shows the effect of the antibiotic at its MIC on both protein synthesis (*a*) and RNA synthesis (*b*) of the highly susceptible *B. cereus*. The inhibition of RNA synthesis appeared to be more immediate and was apparent less than 2 min after treatment with the antibiotic. Protein synthesis, on the

Fig. 3

Fig. 4

Fig. 3. Labelling of indole derivative antibiotics with $[^3\text{H}]$ tryptophan. *X. nematophilus* growing in supplemented minimal medium (Spizizen) were labelled with $2\ \mu\text{Ci}$ ($74\ \text{kBq}$) $[^3\text{H}]$ tryptophan ml^{-1} during the early stationary phase. Antibiotic extraction was done 24 h later. Extracts were chromatographed on analytical silica thin layer plates in three different solvent systems: 1 and 2 were mixtures of methylene chloride/ethyl acetate (respectively 2:8 and 11:9, v/v); system 3 was n-butanol/acetic acid/water (11:5:4, by vol.). The dried chromatograms were subjected to fluorography and autoradiography. Comigration of antibiotic activity with maximal radioactive incorporation was verified by comparing the two R_F values. The data shown are representative of three similar studies. The antibiotic spots are indicated by dashed circles. The actual radioactive counts (c.p.m.) at areas corresponding to the antibiotic spot (radioactivity incorporated into the antibiotic) were: solvent 1, 964; solvent 2, 3177; solvent 3, 3692.

Fig. 4. Labelling of indole derivative antibiotics with $[\text{side-chain-1-}^{14}\text{C}]$ tryptophan and $[\text{side-chain-3-}^{14}\text{C}]$ tryptophan. *X. nematophilus* growing in supplemented minimal medium (Spizizen) were labelled with $0.5\ \mu\text{Ci}$ ($18.5\ \text{kBq}$) of either $[\text{side-chain-1-}^{14}\text{C}]$ tryptophan or $[\text{side-chain-3-}^{14}\text{C}]$ tryptophan ml^{-1} during the early stationary phase. Antibiotic extraction was done 24 h later. Extracts were chromatographed in triplicate on analytical silica thin layer plates in n-butanol/acetic acid/water (11:5:4, by vol.), and the dried chromatograms subjected to fluorography and autoradiography. Comigration of antibiotic activity with maximal radioactive incorporation was verified by comparing the two R_F values. The location or R_F of antibiotic activity is indicated by an asterisk. Lane 1: $[\text{side-chain-1-}^{14}\text{C}]$ tryptophan-labelled extract; lane 2: $[\text{side-chain-3-}^{14}\text{C}]$ tryptophan-labelled extract. The actual radioactive counts (c.p.m., after correction for background counts) on representative chromatograms at the spots corresponding to the R_F of antibiotic activity are: C-1 tryptophan, 548; C-3 tryptophan, 9322.

other hand, was not affected until after 5 min incubation. The rapid inhibition of RNA synthesis caused by the antibiotic was also confirmed using less susceptible bacteria such as *E. coli* (Fig. 6). Again, the effect of the antibiotic on protein synthesis was gradual and not evident until after 5 min indicating that RNA synthesis, rather than protein synthesis, was the primary target of the antibiotic. The antibiotic did not cause any ap-

preciable change in DNA synthesis in either bacterium (data not shown).

Effect on intracellular nucleotide levels

The severe inhibition of RNA and, to a lesser extent, protein synthesis in susceptible bacteria within only a few minutes of treatment with the antibiotics suggested that

Table 1. MIC and comparative growth inhibitory effect of the indole derivative antibiotic and some commercially available antibiotics

The indole derivative antibiotic was purified from crude extracts by chromatography on silica thin layer plates, in n-butanol/ethanol/water (5:1:4, by vol.) Abbreviations: Ery, erythromycin; Tet, tetracycline; Strep, streptomycin; Chlor, chloramphenicol; Indol, indole derivative antibiotic.

	Diameter of inhibition zones for various antibiotics (mm)								MIC* ($\mu\text{g ml}^{-1}$) Indol
	Ery (15 μg)	Tet (30 μg)	Strep (10 μg)	Kan (30 μg)	Chlor (30 μg)	Indol (1 μg)	Indol (2 μg)	Indol (6 μg)	
<i>B. cereus</i>	25	14	15	9	16.5	17	24	26	1.5
<i>E. coli</i> JC355	19	30	0	15	18.5	9	14	21	7.0
<i>E. coli</i> NF161	7	16	10.5	13.5	22	9	11	14	10.0
<i>E. coli</i> NF162	8.5	14.5	9.5	15	17.5	0	0	0	> 100.0
<i>M. luteus</i>	NA	NA	15	11	16	17	27	29	1.0
<i>Proteus vulgaris</i>	0	19	0	14	15.5	11	18	20	7.0
<i>S. marcescens</i>	9	0	8.5	14.5	NA	8	9	14	6.0
<i>P. aeruginosa</i>	0	13	9	0	0	7	7	9	9.0
<i>P. fluorescens</i>	0	16	0	11	0	8	9	12	7.5
<i>P. fragilis</i>	10	25	8	20	13	8	9	12	7.5
<i>P. putida</i>	0	14	0	13	7	12	14	16	2.5

NA, Not assayed.

* Average of three assays for each bacterium. Cells in the exponential phase of growth were diluted to 10^5 – 10^6 viable cells ml^{-1} . Results were read 18–19 h after addition of the indole derivative antibiotic.

these effects could be produced through fast acting, low-molecular-mass regulatory compounds. Furthermore, the rapid inhibition of RNA synthesis was similar to that produced by increased levels of ppGpp during amino acid starvation. Hence, the effect of the antibiotics on intracellular levels of this and other nucleotides was studied in bacteria such as *E. coli*, *Pseudomonas putida*, and *Proteus vulgaris*. Cellular nucleotides were labelled with $\text{H}_3^{32}\text{PO}_4$, followed by nucleotide extraction and chromatography. Autoradiography of chromatographed cell extracts showed that the levels of ppGpp in susceptible bacteria treated with the antibiotic increased substantially when compared to untreated bacteria (Fig. 7). This effect was apparent when bacteria were treated with MICs of the antibiotic (Fig. 7, lanes 2 and 5). Higher concentrations of the antibiotic produced a corresponding increase in ppGpp accumulation (Fig. 7, lanes 3 and 6).

To confirm that ppGpp induction is an obligatory step in the inhibition of RNA synthesis by the indole antibiotic, its effect on the isogenic stringent and relaxed pair of *E. coli* K12 strains, NF161 and NF162 respectively, was investigated. As with the other susceptible bacteria, the antibiotic produced a concentration-dependent increase in ppGpp levels only in the stringently regulated NF161 (Fig. 8, lanes 1–3). The relaxed NF162 strain (which was not susceptible to the antibiotic) showed no detectable increase in ppGpp concentration upon antibiotic treatment, even at concentrations as high as $3 \times \text{MIC}$ for NF161 (Fig. 8, lanes 4–6). In all susceptible bacteria, the enhanced accumulation of

ppGpp was accompanied by a simultaneous decrease in the levels of GTP (Figs 7 and 8). Autoradiography of two-dimensional chromatograms confirmed that there was a preferential accumulation of ppGpp in all susceptible bacteria treated with the antibiotic, but not in the relaxed strain of *E. coli* (data not shown).

Discussion

Previous investigations have indicated that members of the genus *Xenorhabdus* are physiologically and biochemically well equipped for their unique environmental niche as symbionts of entomopathogenic nematodes (Khan & Brooks, 1977; Lysenko & Weiser, 1974; Neilson *et al.*, 1988; Poinar, 1966; Poinar & Thomas, 1966, 1967). Several different secondary metabolites produced by members of this genus (Couche & Gregson, 1987; Couche *et al.*, 1987; Gregson & McInerney, 1985; Neilson *et al.*, 1988; Paul *et al.*, 1981; Rhodes *et al.*, 1983; Richardson *et al.*, 1988; Schmidt *et al.*, 1988) contribute to the success of the symbiotic relationship within the microenvironment of the insect host. Among these, the antibiotics are considered to be of special significance to the maintenance of the mutualistic relationship between the bacteria and the entomogenous nematodes which act as their vectors. Hence, it is important to understand how these natural antibiotics inhibit the wide variety of micro-organisms normally encountered in such an environment, and to determine ways of increasing the antibiotic production. To our knowledge, this is the first study that has focused on the

Fig. 5

Fig. 6

Fig. 5. Inhibition of macromolecular synthesis in *B. cereus* by indole derivative antibiotics. The effect of these antibiotics on accumulation of protein and RNA was studied by pretreating exponentially growing cells in supplemented Hershey's Tris minimal medium with $1.5 \mu\text{g}$ of indole derivative antibiotic ml^{-1} 5 min before addition of $2 \mu\text{Ci}$ (74 kBq) $[^3\text{H}]\text{leucine}$ (a) or $0.4 \mu\text{Ci}$ $[^{14}\text{C}]\text{uracil}$ ml^{-1} (b), respectively. TCA-precipitable counts of 1 ml aliquots taken at different times were collected on Millipore filters, as detailed in Methods. Values represent the average of two assays. (a) Protein synthesis; (b) RNA synthesis. $\square$, Untreated control; $\blacksquare$, bacteria treated with antibiotic.

Fig. 6. Inhibition of macromolecular synthesis of *E. coli* K12 strain JC355 by indole derivative antibiotics. Conditions for this experiment are identical to those outlined in Fig. 5, except that cells were treated with approximately $7 \mu\text{g}$ indole derivative antibiotics ml^{-1} . Values represent the average of two assays. (a) Protein synthesis; (b) RNA synthesis. $\square$, Untreated control; $\blacksquare$, bacteria treated with antibiotic.

mechanism of activity of natural indole derivative antibiotics produced by *X. nematophilus*. Here, we have shown that these antibiotics suppress growth of a wide range of bacteria by increasing the intracellular level of the regulatory nucleotide (ppGpp) in the micro-organisms, which in turn leads to severe inhibition of net RNA synthesis. In addition, our studies shed some light on the physiology of antibiotic biosynthesis in *X. nematophilus*.

With regard to the activity spectrum of these antibiotics, our investigation shows that moderate concentrations of the purified indole derivative antibiotic are effective against both Gram-positive and Gram-negative bacteria. Our results are largely in agreement with those of Akhurst (1982) who reported that phase I *X. nematophilus* inhibited the growth of all Gram-positive and most Gram-negative bacteria used in his study.

The mechanism of bacterial growth inhibition adopted by this group of antibiotics is unusual, but apparently very effective. It is known that actively growing bacterial

cells maintain a relatively narrow range of ppGpp concentrations – the so-called basal level concentrations. Perturbations that result in a deviation from this established ppGpp concentration range usually affect the rate of bacterial growth (Lagosky & Chang, 1981). In our studies, we have shown that growth inhibition in susceptible bacteria is due to the antibiotic-induced accumulation of high levels of ppGpp, which leads to an almost immediate inhibition of RNA accumulation. Strong support for this conclusion comes from the fact that within a few minutes of antibiotic treatment, all bacteria that were susceptible to the indole antibiotic experienced an increased accumulation of ppGpp, and a concomitant reduction in total RNA synthesis. As expected, the degree of inhibition of RNA synthesis correlates to the susceptibility of bacteria to the antibiotic (Figs 5b and 6b; Table 1). The delay in the inhibition of protein synthesis that was noticed in our studies (Figs 5a and 6a) suggests that it is a secondary effect of the inhibition of RNA synthesis.

Fig. 7

Fig. 8

Fig. 7. Effect of indole derivative antibiotics on intracellular levels of ppGpp in *Pseudomonas putida* and *Proteus vulgaris*. Cells growing exponentially at 31 °C in Hershey's Tris minimal medium supplemented with 0.33 mM-phosphate were labelled with 15 μCi (555 kBq) $\text{H}_3^{32}\text{PO}_4 \text{ ml}^{-1}$ for at least one generation. Samples (0.8 ml) of the labelled cultures were treated with 3 or 6 μg of indole derivative antibiotics ml^{-1} , quickly vortexed to disperse the antibiotic, and incubated for 7 min as described in Methods. Radioactive incorporation was stopped by immersion of the culture tubes in ice. Formic acid extraction of the radiolabelled nucleotides from cell pellets was performed as outlined in Methods. One-dimensional ascending chromatography of the nucleotide extract and the relevant unlabelled standards was carried out using 1.5 M- KH_2PO_4 (pH 3.4). Lanes 1 and 4, untreated controls; lanes 2 and 5, 3 μg antibiotic ml^{-1} ; lanes 3 and 6, 6 μg antibiotic ml^{-1} .

Fig. 8. Effect of indole derivative antibiotics on the intracellular levels of ppGpp in *E. coli* K12 strains NF161 (lanes 1–3) and NF162 (lanes 4–6). Cells growing exponentially at 37 °C in supplemented Hershey's Tris minimal medium with 0.33 mM-phosphate were labelled with 15 μCi (555 kBq) $\text{H}_3^{32}\text{PO}_4 \text{ ml}^{-1}$ for at least one generation. Samples (0.8 ml) of the labelled cultures were treated with 5 or 10 μg of indole derivative antibiotics ml^{-1} , quickly vortexed to disperse the antibiotic, and incubated for 7 min as described in Methods. Radioactive incorporation was stopped by immersion of the culture tubes in ice. Lanes 1 and 4, untreated controls; lanes 2 and 5, 5 μg antibiotic ml^{-1} ; lanes 3 and 6, 10 μg antibiotic ml^{-1} .

It should be noted that in all susceptible bacteria, the antibiotic-induced elevated levels of ppGpp correlated with a decrease in GTP. This was to be expected, since GTP is a precursor to ppGpp (Lipmann & Sy, 1976; Sy & Lipmann, 1973). Furthermore, the antibiotic did not significantly increase ppGpp levels in the relaxed *E. coli* strain NF162 (Fig. 8, lanes 4–6), which was found to be resistant to growth inhibition at concentrations of the antibiotics 5–7 $\times$ the MIC of its isogenic stringent partner, strain NF161 (Table 1). These data strongly favour our suggestion that only bacteria capable of responding to appropriate stimuli by elevating their levels of ppGpp are susceptible to the indole antibiotics. Our studies using the chemically synthesized indole

derivative antibiotics have confirmed this conclusion (unpublished data). The fact that a wide range of susceptible bacteria responded in this way (Fig. 7, Fig. 8, lanes 1–3) indicates that this phenomenon is not an isolated incidence. This strongly implies that the increase in the levels of ppGpp accumulation beyond the established physiological (basal) levels of the bacteria is the mechanism of antibiotic action. Interestingly, the antibiotic-susceptible bacterium, *Pseudomonas putida*, has not previously been reported to employ ppGpp as a regulatory nucleotide, while *Proteus vulgaris* has only recently been shown to undergo ppGpp-mediated macromolecular regulation (Sundar & Chang, 1992). In our view, this provides evidence for ppGpp being a more

common and prevalent regulator of bacterial metabolism than suggested by the current literature on this topic.

From a teleological perspective, it may be speculated that the *Xenorhabdi* have adopted such a means of controlling the growth of other bacteria in order to be successful in their symbiotic lifestyle which involves intensely competitive environments such as the insect gut and carcass. Results from our investigations of the mode of action of hydroxy-stilbene antibiotics produced by a related species, *X. luminescens* (Paul *et al.*, 1981) suggest that these bacteria use a similar antibacterial control mechanism (Sundar & Chang, 1992). It may be surprising that two structurally dissimilar antibiotics (Paul *et al.*, 1981) share such a specific mechanism of action. However, given the special role of the *Xenorhabdi* in the life cycle of insect-parasitic nematodes (Khan & Brooks, 1977; Poinar, 1966; Poinar & Thomas, 1966), it is understandable that the two related species of bacteria have evolved a mechanism by which they can produce rapid and severe growth inhibition of other competing bacteria.

We have also made an effort to understand the physiology of antibiotic production in *X. nematophilus*, largely as a means of improving antibiotic yield. The structure of the indole antibiotics implies that tryptophan may function as a precursor in their biosynthesis. The enhanced production of the indole antibiotics upon addition of tryptophan as well as the strong incorporation of radiolabelled tryptophan into the antibiotic provide proof for this suggestion. The much higher incorporation of [*side-chain-3-¹⁴C]tryptophan as compared to that of [*side-chain-1-¹⁴C]tryptophan in our antibiotic radio-labelling assays (Fig. 4) suggests that the process of antibiotic biosynthesis could involve removal of the C-1 carboxy terminal carbon of the indole side-chain moiety. In this context, it is interesting that there is some incorporation of radiolabelled leucine into the antibiotic (unpublished observations), and that supplementation of the growth medium with either or both of the amino acids leucine and isoleucine increases antibiotic yield. These data suggest the possibility that the side chains of hydrophobic amino acids (isoleucine, leucine and valine) may be used in the biosynthesis of at least some of the side chain structural components of the antibiotic.**

In conclusion, the ability of the indole antibiotic to cause severe growth inhibition in a fairly wide variety of bacteria through a novel mechanism of activity makes this antibiotic a potential subject for further development. The unique response to indole antibiotic treatment suggests that small amounts of the antibiotic may be effectively used to study the accumulation of ppGpp and the associated pleiotropic effects on metabolism in bacteria. The involvement of ppGpp in the

antibiotic mechanism also provides an additional example of how this regulatory system can be easily manipulated for control of bacterial growth.

We thank Tom Punnet and Eun Ran Suh for helpful suggestions, and Barbara Bachmann for supplying us with *E. coli* K12 strains NF161 and NF162. We also thank Grant Krow, Yong Kim, Aileen Resnick, and Abimbola Aina for providing us with the chemically synthesized indole antibiotics.

References

- AKHURST, R. J. (1980). Morphological and physiological dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. *Journal of General Microbiology* **121**, 303–309.
- AKHURST, R. J. (1982). Antibiotic activity of *Xenorhabdus* spp., bacteria symbiotically associated with insect pathogenic nematodes of the families *Heterorhabditidae* and *Steinernematidae*. *Journal of General Microbiology* **128**, 3061–3065.
- AKHURST, R. J. & BOEMARE, N. E. (1988). A numerical taxonomic study of the genus *Xenorhabdus* and proposed elevation of the subspecies of *X. nematophilus* to species. *Journal of General Microbiology* **134**, 1835–1845.
- BEDDING, R. A. (1981). Low cost *in vitro* mass production of *Neoaplectana* and *Heterorhabditis* species (Nematoda) for field control of insect pests. *Nematologica* **27**, 109–114.
- BEDDING, R. A. (1984). Large scale production, storage and transport of the insect parasitic nematodes *Neoaplectana* spp. and *Heterorhabditis* spp. *Annals of Applied Biology* **104**, 117–120.
- BEDDING, R. A. & MOLYNEUX, A. S. (1982). Penetration of insect cuticle by infective juveniles of *Heterorhabditis* spp. (*Heterorhabditidae*: Nematoda). *Nematologica* **28**, 354–359.
- BOEMARE, N. E. & AKHURST, R. J. (1988). Biochemical and physiological characterization of colony form variants in *Xenorhabdus* spp. (*Enterobacteriaceae*). *Journal of General Microbiology* **134**, 751–761.
- BURMAN, M. (1982). *Neoaplectana carpocapsae*: toxin production by axenic insect parasitic nematodes. *Nematologica* **28**, 62–70.
- CASHEL, M. (1969). The control of ribonucleic acid synthesis in *Escherichia coli*. IV. Relevance of unusual phosphorylated compounds from amino acid starved stringent strains. *Journal of Biological Chemistry* **244**, 3133–3141.
- CHANG, F. N., CHANG, C. N. & PAIK, W. K. (1974). Methylation of ribosomal proteins in *Escherichia coli*. *Journal of Bacteriology* **120**, 651–656.
- COUCHE, G. A. & GREGSON, R. P. (1987). Protein inclusions produced by the entomopathogenic bacteria *Xenorhabdus nematophilus* subsp. *nematophilus*. *Journal of Bacteriology* **169**, 5279–5288.
- COUCHE, G. A., LEHRBACH, P. R., FORAGE, R. G., COONEY, G. C., SMITH, D. R. & GREGSON, R. P. (1987). Occurrence of intracellular inclusions and plasmids in *Xenorhabdus* spp. *Journal of General Microbiology* **133**, 967–973.
- GALLANT, J., SHELL, L. & BITTNER, R. (1976). A novel nucleotide implicated in the response of *Escherichia coli* to energy source downshift. *Cell* **7**, 75–84.
- GOTZ, P., BOMAN, A. & BOMAN, H. G. (1981). Interactions between insect immunity and an insect-pathogenic nematode with symbiotic bacteria. *Proceedings of the Royal Society* **B212**, 333–350.
- GREGSON, R. P. & MCINERNEY, B. V. (1985). Xenocoumacins. Australian Patent PCT/AU85/00215.
- KHAN, A. & BROOKS, W. (1977). A chromogenic bioluminescent bacterium associated with the entomophilic nematode *Chromonema heliothidis*. *Journal of Invertebrate Pathology* **29**, 253–261.
- LAGOSKY, P. A. & CHANG, F. N. (1978). The extraction of guanosine 5'-diphosphate, 3'-diphosphate (ppGpp) from *Escherichia coli* using low pH reagents: a reevaluation. *Biochemical and Biophysical Research Communications* **84**, 1016–1020.
- LAGOSKY, P. A. & CHANG, F. N. (1981). Correlation between RNA synthesis and basal level guanosine 5'-diphosphate 3'-diphosphate in

- relaxed mutants of *Escherichia coli*. *Journal of Biological Chemistry* **256**, 11651–11656.
- LEAVITT, R. I. & UMBARGER, H. E. (1962). Isoleucine and valine metabolism in *Escherichia coli*. XI. Valine inhibition of the growth of *Escherichia coli* strain K12. *Journal of Bacteriology* **83**, 624–630.
- LIPMANN, F. & SY, J. (1976). The enzymatic mechanism of guanosine 5',3'-polyphosphate synthesis. In *Progress in Nucleic Acid Research and Molecular Biology*, vol. 17, pp. 1–4. Edited by W. E. Cohn. New York: Academic Press.
- LOWEN, P. C. (1976). Novel nucleotides isolated from *Escherichia coli* isolated and partially characterized. *Biochemical and Biophysical Research Communications* **70**, 1210–1218.
- LYSENKO, O. & WEISER, J. (1974). Bacteria associated with the nematode *Neoaplectana carpocapsae* and the pathogenicity of this complex for *Galleria mellonella* larvae. *Journal of Invertebrate Pathology* **24**, 332–336.
- NEALSON, K. H., SCHMIDT, T. M. & BLEAKLEY, B. (1988). Luminescent bacteria: symbionts of nematodes and pathogens of insects. In *Cell to Cell Signals in Plant, Animal and Microbial Symbiosis. NATO ASI series*, vol. H17, pp. 101–123. Edited by S. Scannerini. Berlin: Springer-Verlag.
- PAUL, V. J., FRAUTSCHY, S., FENICAL, W. & NEALSON, K. H. (1981). Antibiotics in microbial ecology: isolation and structure assignment of several new antibacterial compounds from the insect-symbiotic bacteria *Xenorhabdus* spp. *Journal of Chemical Ecology* **7**, 589–597.
- POINAR, G. O. (1966). The presence of *Achromobacter nematophilus* in the infectious stage of a *Neoaplectana* sp. (*Steinernematidae: Nematoda*). *Nematologica* **12**, 105–108.
- POINAR, G. O. & THOMAS, G. M. (1966). Significance of *Achromobacter nematophilus* Poinar and Thomas (Achromobacteraceae: Eubacteriales) in the development of the nematode, DD-136 (*Neoaplectana* sp. *Steinernematodae*). *Parasitology* **56**, 510–514.
- POINAR, G. O. & THOMAS, G. M. (1967). The nature of *Achromobacter nematophilus* as an insect pathogen. *Journal of Invertebrate Pathology* **9**, 510–514.
- POINAR, G. O., THOMAS, G. M. & HESS, R. (1977). Characteristics of the specific bacteria associated with *Heterorhabditis bacteriophora* (*Heterorhabditidae: Rhabditida*). *Nematologica* **23**, 97–102.
- RHODES, S. H., LYONS, G. R., GREGSON, R. P., AKHURST, R. J. & LACEY, M. J. (1983). Xenorhabdin antibiotics. Australian Patent PCT/AU83/00156.
- RICHARDSON, W. H., SCHMIDT, T. M. & NEALSON, K. H. (1988). Identification of an anthraquinone pigment and a hydroxystilbene antibiotic from *Xenorhabdus luminescens*. *Applied and Environmental Microbiology* **54**, 1602–1605.
- SCHMIDT, T. M., BLEAKLEY, B. & NEALSON, K. H. (1988). Characterization of an extracellular protease from the insect pathogen *Xenorhabdus luminescens*. *Applied and Environmental Microbiology* **54**, 2793–2797.
- SPIZIZEN, J. (1958). Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. *Proceedings of the National Academy of Sciences of the United States of America* **44**, 1072–1078.
- SUNDAR, L. & CHANG, F. N. (1992). The role of guanosine 3',5'-bispyrophosphate in mediating antimicrobial activity of the antibiotic 3,5-dihydroxy-4-ethyl-*trans*-stilbene. *Antimicrobial Agents and Chemotherapy* **36**, 2645–2651.
- SY, J. & LIPMANN, F. (1973). Identification of the synthesis of MSI as insertion of a phosphorylated group into the 3'-position in guanosine 5'-diphosphate. *Proceedings of the National Academy of Sciences of the United States of America* **70**, 306–309.
- THOMAS, G. M. & POINAR, G. O. (1979). *Xenorhabdus* gen. nov., a genus of entomopathogenic, nematophilic bacteria of the family *Enterobacteriaceae*. *International Journal of Systematic Bacteriology* **29**, 352–360.