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ORIGINAL PAPER

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Biodegradability of end-groups of the biocide polyhexamethylene biguanide (PHMB) assessed using model compounds

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Abstract Polyhexamethylene biguanide (PHMB), a biocide used in a wide variety of disinfection and preservation applications, is a polydisperse mixture in which the end-groups may be any combination of amine, guanidine and cyanoguanidine. Using PHMB model compounds (1,6-diaminohexane; 1,6-diguanidinohexane; 1,6-di(cyanoguanidino)hexane; 4-guanidinobutyric acid), we have determined the biodegradation characteristics of each end-group in several strains of bacteria isolated for their ability to utilise PHMB as a sole source of nitrogen. Bacteria were screened for growth at the expense of each model compound (at non-inhibitory concentrations) as sole nitrogen source. None of the isolated bacteria was capable of utilising a cyanoguanidine end-group as growth substrate, whereas several bacteria were shown to utilise amine or guanidine end-groups. In particular, a strain of *Pseudomonas putida* was capable of extensive growth with 1,6-diguanidinohexane as a sole nitrogen source, with complete removal of guanidine groups from culture medium within 2 days, and with concomitant formation of unsubstituted urea, which in turn was also utilised by the organism. We conclude that whilst amine and guanidine end-groups in PHMB are likely to be susceptible to biodegradation, cyanoguanidine end-groups are likely to be recalcitrant.

Keywords Biodegradation · Biocide · Cyanoguanidine · Guanidine · Polyhexamethylene biguanide

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Introduction

Polyhexamethylene biguanide (PHMB), first synthesised by Rose and Swain [24], is a cationic polymeric biocide based upon biguanide chemistry. Although compounds containing the biguanide unit have found utility as hypoglycemic agents [16], PHMB and related compounds such as chlorhexidine [25] and alexidine [11] are used primarily as biocides. Indeed, PHMB is utilised in a wide variety of antibacterial applications including industrial disinfection, preservation of cosmetics and as an antibacterial treatment for textiles.

Preparations of PHMB are polydisperse mixtures of polymeric biguanides (Fig. 1), with a weighted average number (n) of 12 repeating hexamethylene biguanide units, ranging from $n = 2$ to $n = 40$. The heterogeneity of the molecule is increased further by the presence of either amine, or cyanoguanidine or guanidine end-groups in any combination at the terminal positions of each chain (Fig. 1). The basicity of the biguanide group ($pK_a = 10.96$ [13]) means that each biguanide unit carries a positive charge at physiological pH. Consequently, PHMB is highly adsorptive to anionic surfaces such as bacterial cell walls and indeed its mechanism of biocidal action is considered to be damage to the cytoplasmic membrane [7]. Recent work [1] has shown that at lower concentrations, PHMB may exert its bacteriostatic effects at least partly by a strong cooperative interaction with (polyanionic) nucleic acids.

Currently, there exists a requirement to determine the environmental fate and behaviour (including biodegradation) of biocides placed on the market in the European Union, as detailed in the Biocidal Products Directive, Chapter 2 [14]. As biocides are by nature toxic to microorganisms, determination of biocide biodegradability is hampered by inhibition or destruction of potential biodegrading microorganisms. For example, the failure of attempts to isolate significant numbers of microorganisms capable of biodegradation of quaternary ammonium compounds [12, 17] was probably due

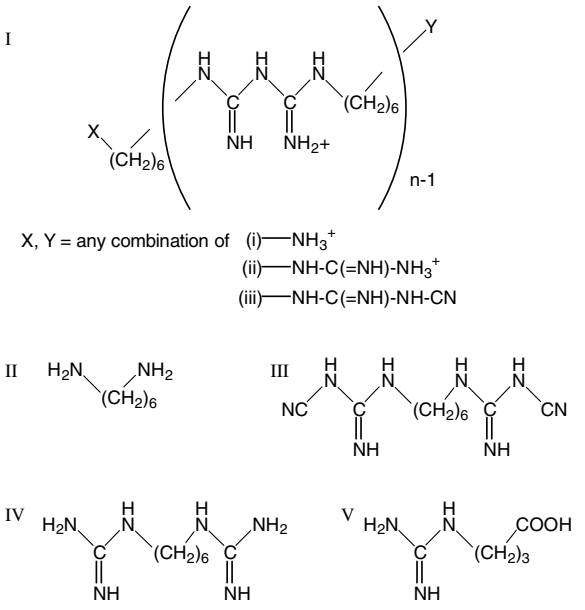

Fig. 1 Molecular structure of polyhexamethylene biguanide (PHMB) and model compounds. *I*, polyhexamethylene biguanide (PHMB), indicating end-groups X and Y as any possible combination of (i), amino; (ii), guanidino; (iii), cyanoguanidino; *II*, 1,6-diaminohexane (DAH); *III*, 1,6-di(cyanoguanidino)hexane (DCGH; *N*′,*N*″-bis(*N*-cyanoamidino)-1,6-diaminohexane); *IV*, 1,6-diguanidinohexane (DGH); *V*, 4-guanidinobutyric acid (GBA)

to the biocidal concentrations employed during the experimentation. In addition, because cationic biocides such as PHMB are highly adsorptive on many solids [15], it is critical that a distinction is made between cationic biocide losses from solution due to biodegradation and adsorption mechanisms [30].

Hence, the study of PHMB biodegradability is confounded by its structural complexity and heterogeneity in terms of chain length and end-groups, its biocidal nature and its propensity for adsorption. To alleviate some of the technical problems associated with determining biodegradability of the molecule, we adopted a reductive approach by using model compounds to assess potential for biodegradation of specific structural motifs. This strategy has been employed previously to study biodegradation of complex structures such as lignin [20, 21] and humic acids [8]. Moreover, it is recognised that the presence of certain structural moieties in a molecule that are more susceptible to microbial attack will promote biodegradation [5]. In particular for polymeric molecules, biodegradation of 3-hydroxybutyrate oligomers has been shown to be dependent on end-group identity [6]. In order to explore the effect of end-group structure in determining biodegradability of PHMB, we tested model compounds, based on the hexamethylene chain of PHMB and terminated with either amine, guanidine or cyanoguanidine end-groupings, for their ability to support growth of microorganisms isolated from enrichment culture in which PHMB was the sole source of nitrogen. The model compounds chosen were (see Fig. 1) 1,6-diaminohexane (DAH), 1,6-diguanidinohexane (DGH), 1,6-di(cyanoguanidino)hexane (DCGH), and 4-guanidinobutyric acid (GBA). These compounds all display lower biocidal activity and decreased propensity for adsorption than PHMB but retained the functional groups of interest that are present in the termini of normal PHMB molecules (Fig. 1).

Materials and methods

Chemicals

Polyhexamethylene biguanide and 1,6-di(cyanoguanidino)hexane (DCGH) were obtained from Avecia (Grangemouth, UK). 1,6-Diguanidinohexane (DGH; as the sulphate salt) was synthesised in the research laboratories of Arch Chemicals (Manchester, UK). The purity of DGH was established by NMR spectroscopy, ES mass spectrometry and elemental analysis. 1-Amino-4-guanidinobutane (agmatine, sulphate salt), 1,4-diaminobutane dihydrochloride (DAB), 1,6-diaminohexane (DAH), 1,4-diguanidinobutane (DGB, arcaine, sulphate salt) and 4-guanidinobutyric acid (GBA) were supplied by Sigma-Aldrich (Gillingham, UK). Working solutions of PHMB, DAH and GBA were prepared in MilliQ (type II, 18 Mohm) high purity water. DGH and DCGH were dissolved in warm 0.075 M HCl and in dimethyl sulphoxide (DMSO), respectively. All other chemicals were obtained from Fisher Scientific (Loughborough, UK) and were of the highest purity available.

Culture media

In liquid culture, bacteria were generally grown in basal salts medium amended with defined carbon and nitrogen sources as required. Nitrogen-free basal salts solution (1× strength) contained the following (per litre), K_2HPO_4, 3.5 g; KH_2PO_4, 1.5 g; NaCl, 0.5 g; $MgSO_4$, 0.12 g and 1 ml of trace elements solution. Trace elements solution contained (per litre), $Na_2B_4O_7$, 0.57 g; $FeCl_3{\cdot}6H_2O$, 0.24 g; $CoCl_2{\cdot}6H_2O$, 0.04 g; $CuSO_4{\cdot}5H_2O$, 0.06 g; $MnCl_2{\cdot}4H_2O$, 0.03 g; $ZnSO_4{\cdot}7H_2O$, 0.31 g and $Na_2MoO_4{\cdot}2H_2O$, 0.03 g. R2A agar was used as a growth medium for agar plates (B.D., Oxford, UK).

Enrichment and isolation of microorganisms

Microorganisms capable of growth at the expense of PHMB as sole nitrogen source were isolated from activated sewage by enrichment culture. The composition of the initial enrichment culture (250 ml flask size) was 5 ml of 20% (w/v) glycerol solution, 4 ml of 2.25 mg l^{-1} PHMB solution, 10 ml of a 10× strength solution of basal salts medium, 0.4 ml of a washed sewage sediment sample, made up to 100 ml with settled sewage supernatant. The enrichment culture was incubated at 30°C and 180 rpm in an orbital incubator for 7 days.

Sewage sediment was washed to remove dissolved nitrogen compounds, which might otherwise serve as alternative N-sources to PHMB in enrichment cultures. Washed sewage sediment was obtained by subjecting 50 ml of aerated activated sewage (from a municipal sewage treatment works, Coslech, South Wales, UK) to six successive washing cycles each comprising centrifugation (900*g* for 5 min), removal of the supernatant and re-suspension of the pellet in phosphate-buffered saline (PBS; 20 ml of 0.85% (w/v) NaCl/10 mM sodium phosphate, pH 7). The final pellet was re-suspended in 10 ml of PBS. Settled sewage supernatant was obtained by allowing activated sewage to settle under gravity for 2 h, after which the upper layer was collected.

After the initial round of growth, five further rounds of serial sub-cultures were conducted at weekly intervals in 250 ml flasks, each containing 5 ml of 20% (w/v) glycerol solution, 4 ml of 2.25 mg l^{-1} PHMB solution, 90 ml of basal salts medium, and 1 ml of inoculum from the previous culture vessel. In addition, from round two of enrichment, a second enrichment regime was initiated which differed only in that a semi-continuous addition of PHMB was made (4 ml aliquots of 2.25 mg l^{-1} PHMB added at day 2, 4 and 6 of each round of culture).

Isolation of microorganisms was performed by plating out aliquots (0.1 ml) of appropriate dilutions of medium from the final enrichment flasks onto R2A agar plates and incubating at 30°C for 5 days. Isolates distinguished by colony morphology were sub-cultured on fresh R2A plates until axenic. After sufficient growth, strains were preserved for long-term storage at −70°C using the Protect™ microbiological bead storage system (Technical Service Consultants Ltd, Lancs, UK). Short-term storage was achieved on R2A agar slopes, which were stored at room temperature in the dark, and sub-cultured every 2 months.

Identification of microorganisms

A Gram determination for each bacterial isolate was conducted using either traditional methods [9], or using a KOH-based method [22]. For the majority of the isolated microorganisms, template DNA for PCR amplification was prepared by re-suspending a bacterial colony in 0.1 ml of sterile high purity water, centrifuging at 13,000*g* for 2 min, resuspending as before, and heating at 96°C for 5 min. Supernatant from this lysate was removed by centrifugation (13,000*g*, 2 min) and retained as template. In cases where it proved difficult to amplify PCR product, the alternative method of Ausubel et al. [4] was used to isolate template DNA.

Recommended [18] bacterial 16S rRNA gene primers 63f (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and 1387r (5′-GGG CGG WGT GTA CAA GGC-3′), where W is A or T, were obtained from Invitrogen (Paisley, UK) and used to amplify a 1.4 kb 16S rDNA gene fragment. PCR reaction mixtures contained the following components: genomic DNA template (undetermined mass of DNA, 1 µl of template solution used), 1.25 units of *Taq* DNA polymerase (Promega, Southampton, UK), 5 µl of 10× PCR buffer ($MgCl_2$ free), 0.75 mM $MgCl_2$, 2 ng of each primer and 250 µM of each deoxynucleotide triphosphate; reaction volumes were made up to 50 µl with sterile milliQ water. PCR amplification was performed in a Primus PCR Thermo-cycler (MWG-Biotech, Ebersberg, Germany), programmed to perform a denaturing step at 94°C for 2 min, followed by 30 rounds of temperature cycling at 92°C for 20 s, 55°C for 30 s and 75°C for 30 s. A final extension step at 75°C for 5 min was conducted before cooling to 4°C.

The PCR products (4 µl) were examined by agarose gel (1%, w/v) electrophoresis (200 V, 150 mA, 30 min in TBE buffer containing 0.5 µg ml^{-1} ethidium bromide). The remaining PCR products were then purified using a Qiagen QIAquick PCR purification kit (Crawley, UK) according to the manufacturer's instructions. Sequences of PCR products were determined (using the 63f primer and an ABI Prism 377 DNA Sequencer) and compared with known sequences in the EMBL database using a BLASTN search program [2] to identify sequence alignments. When obtained sequences were 97–100% identical to strains present in the EMBL database, the corresponding bacterial designations were made at species level, whereas those that possessed a sequence identity of less than 97% were made at genus level [28].

Determination of maximum non-inhibitory concentration for model compounds

Each bacterial isolate was grown on R2A agar plates at 30°C for 48 h, and confluent biomass was re-suspended in sterile PBS to an attenuance ($D_{590\ nm}$) of 0.5 (2 cm path length). Each bacterial suspension (0.3 ml) was combined with 29.7 ml of basal salts medium containing 10 g l^{-1} glycerol and 0.05 g l^{-1} NH_4Cl as carbon and nitrogen sources, respectively. Aliquots (0.2 ml) of each diluted cell-suspension were added to wells in the first column of a 96-well plate and 0.1 ml aliquots to the remaining wells. Aliquots (0.02 ml) of working solutions of test compound (either DGH, DCGH, DAH or GBA, 3,080 mg N l^{-1}) were added to the first column to produce 280 mg N l^{-1}. Serial (twofold) dilution of each compound was then achieved by sequential transfer of 0.1 ml samples from well to well across each row, with mixing between transfers. Growth of bacteria in microtitre plates amended with test compounds was compared with growth in control plates treated identically except that the initial amendment to the first well was with the corresponding working solution solvent (i.e. water for DAH and GBA; 0.075 M HCl for DGH and DMSO for DCGH). Plates were incubated at 30°C and after 48 h, attenuance ($D_{540\ nm}$) of each well was determined using a Thermomax microtitre plate reader (Molecular Devices, Wokingham, UK) (Fig. 2).

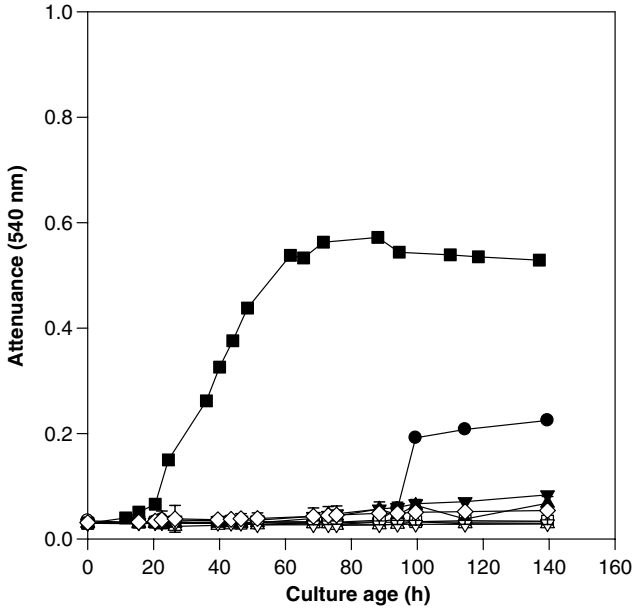

Fig. 2 Growth of PHMB enrichment-culture isolates at the expense of DGH (140 mg N l^{-1}) as sole nitrogen source. Growth was measured as attenuance of the culture medium at 540 nm: *open circle*, strain LPO1; *filled circle*, *E. meningoseptica*; *open square*, *Alcaligenes* sp.; *open triangle*, *C. indologenes*; *filled square*, *P. putida*; *filled triangle*, *K. pneumoniae*; *filled inverted triangle*, *Burkholderia* sp; *open inverted triangle*, *Acinetobacter* sp.; *open diamond*, control (no nitrogen source added) displaying highest recorded attenuance. *Error bars* indicate 95% confidence limits for control readings ($n = 36$)

Growth of enrichment culture isolates on model compounds as sole source of nitrogen

Growth of each bacterial isolate at the expense of either DGH, DCGH, DAH or GBA was determined at a range of concentrations up to 280 mg N l^{-1} in microtitre plates in a manner identical to that used to determine maximum non-inhibitory concentrations (MNICs) for each compound, except that NH_4Cl was omitted from the basal salts medium, thus forcing organisms to utilise model compounds as sources of nitrogen for growth. Control plates lacked all nitrogen sources. Attenuance ($D_{540\ nm}$) was determined over a period of 7 days.

Growth of *Pseudomonas putida* at the expense of DGH

P. putida, isolated in this study from enrichment culture using PHMB as a sole source of nitrogen (see Table 1), was grown in 50 ml of basal salts medium (100 ml flask size), containing glycerol (1%, v/v) and DGH (680 mg l^{-1}; equivalent to 190 mg N l^{-1}) as sole nitrogen source. Cultures were incubated at 30°C and 180 rpm and at intervals samples (0.2 ml) were removed for determination of growth by measurement of attenuance ($D_{540\ nm}$). At the same times, 0.5 ml aliquots of culture medium were removed and retained at −20°C for later chemical analysis.

TLC analysis

Retained culture samples were thawed and centrifuged to remove biomass, and aliquots (0.02 ml) analysed by TLC on silica gel plates (250 μm layer thickness, 60 Å particle size, 20×20 cm, Whatman, Maidstone, UK). The chromatograms were developed using butan-1-ol/acetic acid/H_2O (3:1:1, by vol.) and subsequently dried at 110°C. Compounds were routinely detected by exposing the plates to I_2 vapour.

Determination of urea

After removal of biomass, culture fluid (30 μl) was added to 0.97 ml of H_2O and 1 ml of the working reagent,

Table 1 Identification of microorganisms isolated from enrichment culture, amended with PHMB as a sole source of nitrogen. Isolate designation was conducted by amplification of a 16S rRNA gene fragment, and comparison of the resultant gene sequences to those contained in the EMBL sequence database

Strain	Closest match			Designation of isolate[b]
	Organism	Similarity between test and database sequence[a]	EMBL accession number	
1	Uncultured β-proteobacterium	674/692 (97%)	AB112464	Strain LPO1
2	*Elizabethkingia meningoseptica* strain LDVH 337.01	641/650 (98%)	AY468482	*Elizabethkingia meningoseptica* strain LPO2
3	*Alcaligenes* sp. STC1	599/603 (99%)	AB046605	*Alcaligenes* sp. strain LPO3
4	*Chryseobacterium indologenes*	512/546 (93%)	AY050493	*Chryseobacterium* sp. strain LPO4
5	*Pseudomonas putida* (strain JCM 6156 [ATCC 33015])	623/630 (99%)	D37924	*Pseudomonas putida* strain LPO5
6	*Klebsiella pneumoniae* strain ATCC 13884T	601/622 (97%)	Y17657	*Klebsiella pneumoniae* strain LPO6
7	*Burkholderia* sp. KBC-4	592/609 (97%)	AY769905	*Burkholderia* sp. stain LPO7
8	*Acinetobacter* sp. (strain ATCC 9957) or *Acinetobacter lwoffii* (DSM2403)	456/474 (96%)	Z93442 or X81665	*Acinetobacter* sp. strain LPO8

[a]Calculated as number of bases identical/total number compared
[b]Designated strain is provided at species level where similarity between test and database sequence is 97% or above, and at genus level for similarity of below 97%

and assayed for urea content as described elsewhere [23]. A calibration curve was constructed using standard solutions of urea. Urea was discriminated from N-substituted ureas by measurement of λ_{max} for each sample [3], and comparison of the resultant values to those given by standard samples (1 mM) of urea (non-N substituted) and L-citrulline (N-substituted).

Determination of guanidino compounds

After removal of biomass, culture fluid (35 µl) and H_2O (0.565 ml) were mixed and added to 0.3 ml of 3 M NaOH and 0.6 ml of the assay reagent. After 10 min, 1.5 ml of deionised H_2O was added, and after a further 15 min, the samples were assayed for guanidine-group content as described elsewhere [26]. A calibration curve was constructed by replacing the diluted culture fluid with 0.6 ml aliquots of standard solutions of DGH.

Results

Enrichment and identification of microorganisms

After six rounds of enrichment, five distinct bacterial strains (strains 1–5) were isolated from the single-dose PHMB-enrichment culture, and three other distinct bacterial strains (strains 6–8) were isolated from the semi-continuously dosed PHMB-enrichment culture. All except for strain 1 were Gram negative. Alignment of 16S rRNA gene sequences (of lengths between 474 and 692 base pairs) with those present in the EMBL database resulted in designation of each strain at either species or genus level [28] (Table 1). For strain 8, two sequence alignments with identical scores were given, both for *Acinetobacter* strains. In addition, for strain 1 the highest scoring sequence alignment was for an uncultured β-proteobacterium, which conflicted with the results of Gram staining. Therefore, strain 1 remained unclassified and was designated as strain LPO1.

Maximum non-inhibitory concentration for model compounds

Table 2 shows the bacteriostatic activity of each model compound for each isolate from enrichment culture. Of the model compounds tested, DAH possessed the greatest bacteriostatic activity, which was observed in the range of 70–140 mg l^{-1} total nitrogen. DGH only inhibited the growth of the *Burkholderia* sp. DCGH and GBA did not result in inhibition of growth of any strains in the concentration range tested (0–280 mg N l^{-1}). The MNIC values recorded for each model compound are much higher than those previously reported for PHMB against Gram negative and Gram positive bacteria, which are typically reported in the range of 1–30 mg l^{-1} [19].

Growth of PHMB enrichment-culture isolates on model compounds

Each compound (DAH, DGH, DCGH and GBA) was screened at a range of concentrations up to 280 mg N l^{-1}, for its ability to serve as a sole source of nitrogen for each of the strains isolated using PHMB as a sole source of nitrogen. For comparative purposes, and in order to avoid any complications from inhibition of bacterial growth, Table 3 shows only the highest growth recorded for compounds at concentrations of either 140 mg N l^{-1} or the MNIC, whichever was the lower (see Table 2). All strains except the *Chryseobacterium* isolate grew well on the naturally occurring compound GBA. Three of the isolates also grew on DAH for which natural homologues exist, e.g. 1,4-diaminobutane (putrescine). Of the remaining compounds, DCGH was incapable of supporting the growth of any

Table 2 Maximum non-inhibitory concentrations (MNICs) for each model compound against microorganisms isolated from PHMB enrichment culture. Concentration range was 0–280 mg N l^{-1} for each compound. A value of > 280 mg N l^{-1} is given where no biocidal activity in the concentration range tested was detected

Isolate	MNIC (mg N l^{-1})[a]			
	DAH[b]	DGH	GBA	DCGH
Strain LPO1	> 280	> 280	> 280	> 280
Elizabethkingia meningoseptica strain LPO2	> 280	> 280	> 280	> 280
Alcaligenes sp. strain LPO3	70	> 280	> 280	> 280
Chryseobacterium sp. strain LPO4	140	> 280	> 280	> 280
Pseudomonas putida strain LPO5	140	> 280	> 280	> 280
Klebsiella pneumoniae strain LPO6	> 280	> 280	> 280	> 280
Burkholderia sp. strain LPO7	70	35	> 280	> 280
Acinetobacter sp. strain LPO8	140	> 280	> 280	> 280

[a]MNIC determinations were conducted in duplicate, of which the lowest determined value is reported
[b]Compounds tested were DAH, 1,6-diaminohexane; DGH, 1,6-diguanidinohexane; GBA, 4-guanidinobutyric acid; DCGH, 1,6-di(cyanoguanidino)hexane

Table 3 Growth of PHMB enrichment culture isolates at the expense of selected model compounds

Isolate	Highest attenuance (D_{540}) recorded during growth on substrates added at or below MNIC[a]				
	DAH	DGH	GBA	DCGH	Control[b]
Strain LPO1	0.03	0.03	0.66	0.03	0.05 ± 0.05
Elizabethkingia meningoseptica strain LPO2	0.38	0.23	0.84	0.03	0.06 ± 0.04
Alcaligenes sp. strain LPO3	0.04[c]	0.04	0.37	0.05	0.04 ± 0.01
Chryseobacterium sp. strain LPO4	0.03	0.03	0.03	0.04	0.07 ± 0.08
Pseudomonas putida strain LPO5	0.49	0.57	0.70	0.08	0.06 ± 0.01
Klebsiella pneumoniae strain LPO6	0.54	0.07	0.40	0.04	0.06 ± 0.04
Burkholderia sp. stain LPO7	0.07[c]	0.06[d]	0.52	0.04	0.06 ± 0.01
Acinetobacter sp. strain LPO8	0.04	0.03	0.45	0.04	0.04 ± 0.01

[a]Growth determined at 140 mg N l^{-1} for compounds that displayed an MNIC of 140 or 280 mg N l^{-1} (see Table 2)
[b]Control values are the highest attenuance recorded for control wells where no nitrogen source was added, ±95% confidence limits
[c]Growth determined at MNIC of 70 mg N l^{-1} (Table 2)
[d]Growth determined at MNIC of 35 mg N l^{-1} (Table 2)

strain tested, but DGH was a good substrate for *P. putida* and, to a lesser extent, *E. meningoseptica* (Fig. 2). Growth of *P. putida* on DGH was therefore selected as a system for further study (see below).

Growth of *Pseudomonas putida* at the expense of DGH

Growth of *P. putida* on DGH as a sole source of nitrogen was accompanied by the progressive disappearance of DGH in a TLC analysis (Fig. 3) and appearance of several new spots of high mobility (Fig. 3, tracks 5–8). The new components did not correspond in mobility to any nitrogen-containing standards tested (DAH, 1,4-diaminobutane, 1,4-diguanidinobutane, 1- amino-4-guanidinobutane or urea, tracks 10–14, respectively). Urea-based compounds (R_f 0.6) were identified as being present in samples applied to tracks 4–6 (data not shown) by spraying with a diacetyl-monoxime/antipyrine colorimetric reagent mixture.

The degradation of DGH by *P. putida* and concomitant formation of urea were quantified by colorimetric assays. The decrease in concentration of DGH from 2.27 mM to 0 over a period of 45 h (Fig. 4) confirmed the results of TLC analysis and showed that this period also corresponded to the period of exponential growth, and to the appearance of urea in the growth medium. The urea released into the culture medium was confirmed as lacking N-substitution by the method of Archibald [3]. The concentration of urea was maximal at 45 h, and was rapidly depleted thereafter. The point of maximum concentration corresponded to the time of fastest growth and to the point of exhaustion of DGH substrate. The detection, by TLC, of urea in samples taken at 43.5, 45 and 48 h was completely consistent with data provided by colorimetric assay of urea. In an

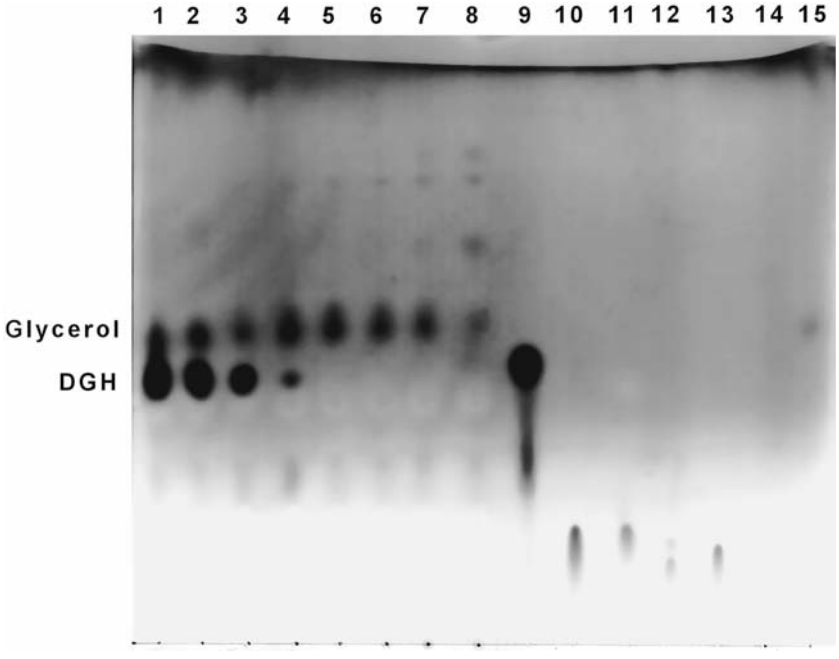

Fig. 3 TLC analysis of DGH degradation in *P. putida* culture medium. Culture samples were removed at various time points from the culture amended with DGH (680 mg l^{-1}) as sole nitrogen source (see Fig. 2), and subjected to TLC analysis (see Materials and methods). Spots were revealed using I_2 vapour. *Tracks 1–8*, 20 µl of DGH-amended *P. putida* culture medium, sampled at 0, 26.5, 39.5, 43.5, 45.0, 48, 52 and 87 h, respectively; *track 9*, standard DGH; *track 10*, DAH; *track 11*, DGB; *track 12*, agmatine; *track 13*, DAB; *track 14*, urea; and *track 15*, glycerol

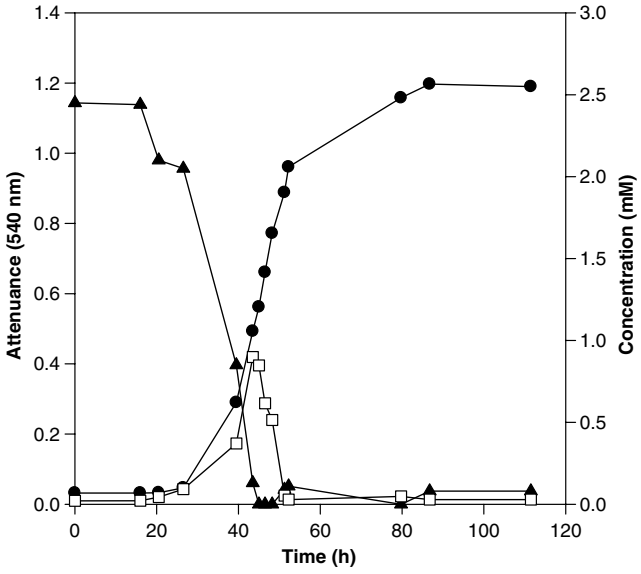

Fig. 4 Biodegradation of DGH (680 mg l^{-1}) as sole nitrogen source by *P. putida*. Bacterial growth (*filled circle*) was determined as culture attenuance at 540 nm; Concentrations of DGH (*filled triangle*) and urea (*open square*) were determined as described in Materials and methods

independent experiment, *P. putida* was shown to be capable of utilising urea as a sole nitrogen source (data not shown).

Discussion

All but one of the bacterial strains (*Chryseobacterium* sp.) used GBA as a sole nitrogen source, and three organisms (*P. putida*, *E. meningoseptica* and *K. pneumonia*) were also capable of using DAH. GBA is formed in fluorescent species of *Pseudomonas* (including *P. putida*) as a central intermediate of the arginine oxidase pathway that converts arginine to 4-aminobutyrate via 2-ketoarginine, 4-guanidinobutyraldehyde and GBA [10]. *P. putida* has also been shown to remove guanidine from GBA via a guanidinebutyrate amidinohydrolase activity [29]. 1,4-Diaminobutane (putrescine), the C4 homologue of DAH, is an intermediate of the arginine decarboxylase pathway, in which arginine is converted to 4-aminobutyrate via 1-amino-4-guanidinobutane (agmatine), *N*-carbamoylputrescine, putrescine, and 4-aminobutyraldehyde. Putrescine utilisation has been detected in *Klebsiella*, *Pseudomonas* (including *P. putida*) and *Aeromonas* spp. [10]. Therefore, given the importance of GBA and diaminoalkanes in bacterial metabolism, it is not surprising that several of the PHMB enrichment culture isolates are capable of growth at the expense of DAH and GBA.

Two of the eight bacterial strains were capable of utilising the guanidine end-group model compound DGH as a sole source of nitrogen, of which *P. putida* displayed the highest growth yield. However, no bacterium was capable of using the cyanoguanidine end-group model compound DCGH. The important implication for degradation of PHMB is therefore that the isolated bacteria are capable of utilising nitrogen from terminal guanidine or amine PHMB end-groups but not from the cyanoguanide end-group. Degradation of bis-guanidino structures has been reported previously, but only for *P. putida* [31], various other *Pseudomonas* sp. [29] and a species of *Rhodococcus* [27]. Therefore, this published evidence is in agreement with the observation that, of the isolates obtained from enrichment culture, *P. putida* is capable of the most extensive growth using DGH as a sole nitrogen source.

A *Rhodococcus* sp. is already known to metabolise DGB (1,4-diguanidinobutane; the shorter, C4 homologue of DGH) via initial deamination of one of the terminal guanidino groups to form carbamoylagmatine, followed by hydrolytic removal of the newly formed carbamoyl group to yield agmatine; this two-step reaction is then repeated on the other guanidino end-group to yield putrescine [27]. In this pathway, no urea is liberated. In contrast, *P. putida* catabolised DGB to agmatine in a single step with formation of urea [31]. To determine which of these mechanisms operates in the degradation of the C6 homologue DGH in our *P. putida* strain isolated for its ability to grow on PHMB, DGH disappearance and urea formation were monitored by TLC and by quantitative colorimetric analysis. *P. putida* removed DGH completely from the culture medium with the concomitant formation of urea in a typical precursor–product relationship. Independent experiments established that urea was able to serve as a sole source of nitrogen in *P. putida*. All of the urea liberated from DGH was released in a non-N-substituted form. All together, these data suggest that *P. putida* metabolises DGH via 1-amino-6-guanidinohexane (the C6 homologue of agmatine) with concomitant formation of urea (Fig. 5), as previously reported for DGB metabolism in *P. putida* [31], and not via the pathway for DGB metabolism reported for a *Rhodococcus* sp., where no urea was liberated [27].

Collectively, the data obtained in the growth experiments indicated that microorganisms isolated for their ability to derive nitrogen for growth from PHMB can obtain nitrogen from amine and guanidine end-group structural moieties, but not from cyanoguanidine end-groups. This implies that the PHMB components with cyanoguanidine end-groups are unlikely to be biodegraded at those termini by microorganisms, whereas components with amine and guanidine end-groups are likely to be readily biodegradable. The mechanism of PHMB degradation has yet to be established. However, if it should proceed via an exocleavage mechanism (i.e. progressive degradation from the ends of the molecules) the presence of cyanoguanidine end-groups may inhibit biodegradation for at least a fraction of the PHMB molecules within the heterogeneous mixture. It is expected that an equal ratio of cyanoguanidine, guanidine and amine end-groupings will be present in PHMB. Therefore, we suggest from this evidence that there may

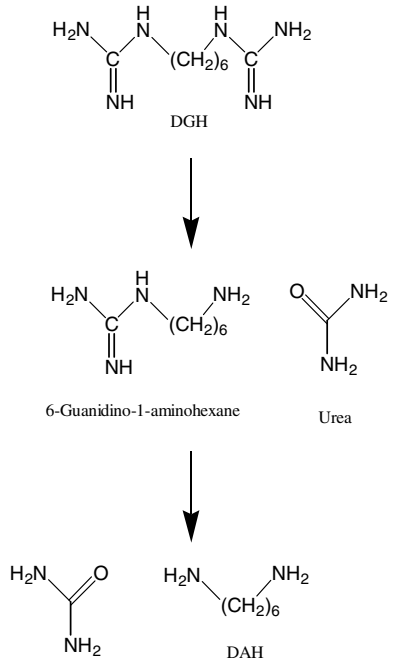

Fig. 5 Proposed biodegradation pathway for DGH

be an opportunity to increase the biodegradability of PHMB by lowering the proportion of cyanoguanidine end-groups in the polymeric mixture.

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