Characterization of biofilm formation by clinical isolates of *Mycobacterium avium*

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Mycobacterium avium is an environmental organism encountered in natural and urban water sources as well as soil. *M. avium* biofilm has recently been identified on sauna walls and in city water pipes and might have a role in the survival of virulent strains in the environment and in the host. To characterize the *M. avium* biofilm, an *in vitro* model was adapted wherein biofilm develops on a PVC surface. Biofilm was detected by staining with crystal violet and visualization by optical microscopy and quantified by A_{570} . *M. avium* strains MAC 101, MAC 100, MAC 104, MAC 109, MAC A5 and MAC 5501 (all isolated from the blood of AIDS patients) were used in the assays. Biofilm formation was dependent on the presence of Ca^{2+} , Mg^{2+} or Zn^{2+} ions in the water, with the maximal effect seen at a concentration of 1 μM . The presence of 2 % glucose and peptone as sources of carbon increased the formation of biofilm, while this was partially inhibited by humic acid. Since sliding motility has been associated with the amount of glycopeptidolipid (GPL), TLC was used to determine the presence of GPL. The supernatant of a biofilm-forming culture induced formation of a stable biofilm and amikacin blocked the establishment of biofilm by *M. avium* strains at subinhibitory concentrations. Bacteria in the biofilm were more resistant to chlorine as well as to exposure to potassium monopersulfate and chloroheximide acetate than were planktonic bacteria. Identification of *M. avium* genes involved in biofilm formation and further studies of the effect of antimicrobials on the establishment of biofilm may identify approaches for inhibiting *M. avium* biofilm formation and colonization.

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INTRODUCTION

Great emphasis has been placed on the study of the interaction between human pathogenic mycobacteria, such as *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium intracellulare* and *Mycobacterium leprae*, and the human host, but few studies have been directed at the interaction of mycobacteria with the natural environment.

M. avium is an opportunistic pathogen that occurs naturally in the environment but can occasionally cause disease in animals and humans, especially in immunocompromised individuals (Inderlied *et al.*, 1993; Falkinham, 1996). *M. avium* is the main cause of bacteraemia and bacterial disseminated infection in AIDS patients, which is particularly difficult to treat (Wolinsky, 1979). Furthermore, *M. avium* is associated with lung disease in patients with

chronic pulmonary pathology such as emphysema and bronchiectasis (Prince *et al.*, 1989).

A considerable percentage of the population has been shown to react to *M. avium* antigens (Bermudez *et al.*, 1989), correlating with the finding that *M. avium* is isolated from environmental sources. More recently, it has been observed that *M. avium* can develop biofilms in urban water systems and on sauna walls (Carson *et al.*, 1978; Falkinham *et al.*, 2001; Schulze-Robbecke & Fischeder, 1989). *M. avium* isolates recovered from water systems and natural water sources have the same DNA fingerprint as those isolated from AIDS patients (von Reyn *et al.*, 1994). In addition, since these bacteria are resistant to chlorination (Taylor *et al.*, 2000) and acidic pH (Bodmer *et al.*, 2000), it can be assumed that they are also common in urban water as planktonic cells, a possibility confirmed in a number of previous studies (Glover *et al.*, 1994; Haas *et al.*, 1983).

Martinez *et al.* (1999) reported that both *Mycobacterium*

Abbreviation: GPL, glycopeptidolipid.

smegmatis and *M. avium* can spread on a soft surface by sliding motility. Genetic analysis of *M. smegmatis* demonstrated that clones with a transposon inserted in transport gene *tmtP*-C and biosynthetic genes *mps* do not express sliding motility and do not form biofilms (Recht *et al.*, 2000). These genes encode enzymes involved in glycopeptidolipid (GPL) biosynthesis.

In order to characterize *M. avium* biofilm and the influence of environmental cues on its formation better, we determined the effect of several conditions on the formation of biofilm.

METHODS

Strains and growth media. *M. avium* strains MAC 101, MAC 100, MAC 104, MAC 109, MAC A5 and MAC 5501 were obtained from the blood of AIDS patients. The strains were identified according to serovar by Robert Good (Centers of Disease Control, Atlanta, GA, USA). Culture stocks were established following isolation and bacteria used in the study were obtained from the stock. Mycobacteria were grown aerobically at 37 °C in Middlebrook 7H9 medium (Difco) containing 10 % oleic acid, albumin, glucose and catalase (OADC; Hardy Scientific). For growth on solid media, bacteria were plated on 7H10 agar and grown at 37 °C.

Biofilm formation and analysis. Biofilm formation was determined as described previously (O'Toole *et al.*, 2000) by seeding 200 µl water, buffer or 7H9 broth containing 1×10^7 bacteria in a PVC plastic 96-well microtitre plate (Falcon 3911, Microtest III flexible assay plate; Becton Dickinson). It was assayed by determining the ability of cells to adhere to the wells as reported previously (Limia *et al.*, 2001). Briefly, after inoculation, plates were incubated at room temperature for 14 days and then 25 µl of a 1 % crystal violet solution was added to each well (this dye stains the cells but not the PVC). The plates were incubated at room temperature for 15 min, rinsed vigorously four times with water, blotted on paper towels and scored for biofilm formation. The crystal violet was solubilized in 95 % ethanol and the A_{570} was determined as described previously (Limia *et al.*, 2001; O'Toole *et al.*, 2000).

Lipid extraction and TLC. GPLs were isolated as described previously (McNeil *et al.*, 1989) with minor modifications. Briefly, bacterial cultures (150 ml) were grown to turbidity, concentrated by centrifugation in a swinging bucket centrifuge (Sorval RT7) at 1000 g for 10 min, resuspended in a small volume of water (10 ml) and lyophilized for 2 h. The dry residue was extracted with 50 ml chloroform/methanol (2 : 1) and non-solubilized material was removed by centrifugation at 1000 g for 15 min. The supernatant was carefully removed and the pellet was washed once with a similar volume of solvent. The combined supernatants were dried by rotation evaporation, resuspended in 50 ml chloroform/methanol/0.2 M NaOH (2 : 1 : 1) and incubated at 50 °C for 30 min to hydrolyse any ester-containing phospholipids. The incubation mixture was subsequently neutralized with glacial acetic acid. Phase separation was induced by addition of more of both the immiscible aqueous (water, 25 ml) and organic (2 : 1 chloroform/methanol; 3–4 ml) phases and the mixture was stored in glass vials.

GPLs were analysed by TLC using normal-phase silica gel chromatographic plates (Analtech) and a solvent system composed of chloroform/methanol/water (30 : 8 : 1). The resolved lipids were developed by iodine staining and subsequently scanned and analysed using the NIH Image software (<http://www.nih.gov>).

Effect of different conditions on biofilm formation. To determine whether the presence of ions or source of carbon had any effect on

biofilm formation, bacteria were incubated in PVC plates in the presence or absence of different concentrations of humic acid, glucose, peptone, 7H9 broth, Ca^{2+} , Zn^{2+} , Mg^{2+} and Fe^{2+} and biofilm formation was determined as described above.

Effect of culture supernatant on biofilm formation. In order to examine whether culture supernatant had any effect on biofilm formation, supernatant obtained from *M. avium* culture in 7H9 broth for 7 days in PVC plates was used. Supernatant was filter-sterilized by passing it through a 2 µm pore filter. Subsequently, 50 µl culture supernatant or 50 µl 7H9 broth (as a control) was mixed with 100 µl water and 1×10^7 bacteria were added. To evaluate the possible role of protein in the formation of *M. avium* biofilm, bacteria were incubated in PVC plates with 4 µg amikacin ml^{-1} , a subinhibitory concentration of the antibiotic that inhibits protein synthesis but not bacterial growth (Bermudez *et al.*, 1992).

Resistance of biofilm to disinfectants. Working solutions of biocides were prepared in distilled water and diluted as desired. Biofilm and planktonic bacteria in 96-well PVC plates were challenged for 30 and 60 min, washed with PBS (planktonic bacteria were centrifuged at 10 000 g for 20 min), serially diluted and plated onto 7H11 agar plates. The biocides were used at concentrations of 21 % potassium monochloride, 2 % chloroheximide acetate and 5 % sodium hypochloride (Bardouniotis *et al.*, 2001).

Statistical analysis. Each experiment was repeated at least four times and the results are expressed as means $\pm$ SD. The experimental results were compared with controls and analysed by the non-parametric Mann–Whitney test; $P < 0.05$ was considered significant.

RESULTS

Biofilm formation by *M. avium* strains

To determine whether the ability to form biofilm differed among several *M. avium* strains, we used four strains obtained from patients and placed them in either water or 7H9 broth. As shown in Table 1, MAC A5 was associated with greater biofilm formation after 14 days than MAC 109, MAC 101 and MAC 104. MAC 100 was the strain that developed the weakest biofilm. Table 1 also shows that *M. avium* strains formed significantly more biofilm on a PVC surface when incubated in water than in 7H9 broth and that the ability to establish a biofilm in 7H9 medium did not differ among strains MAC 101, MAC 109 and MAC A5.

Effect of ions on *M. avium* biofilm formation

To examine whether different concentrations of ions such as Mg^{2+} , Ca^{2+} , Fe^{2+} and Zn^{2+} , encountered in the environment, could affect the ability of *M. avium* strains to form biofilm, we used strain MAC A5 in water in the presence of a range of concentrations of ions. Table 2 shows that the presence of Ca^{2+} , Mg^{2+} or Zn^{2+} was associated with increased biofilm formation compared with growth in water alone and that the amount of biofilm formed in the presence of concentrations of Ca^{2+} , Mg^{2+} and Zn^{2+} from 0.1 to 10 µM did not vary significantly.

Effect of the source of carbon on biofilm formation

Because *M. avium* in the environment is exposed to different

Table 1. Biofilm formation in water and 7H9 medium

Bacteria were incubated in different media in a PVC 96-well plate for 14 days. Biofilm formation was evaluated at days 1, 4, 7 and 14 as described in Methods. Values are means $\pm$ SD A_{570} readings from three experiments (multiplied by 1000).

Strain	Medium	Day 1	Day 4	Day 7	Day 14
MAC 100	Water	42 $\pm$ 3	96 $\pm$ 38	216 $\pm$ 54*	436 $\pm$ 45*
	7H9	44 $\pm$ 8	68 $\pm$ 11	87 $\pm$ 10	124 $\pm$ 28
MAC 101	Water	46 $\pm$ 4	180 $\pm$ 41*	424 $\pm$ 26*	691 $\pm$ 25*
	7H9	47 $\pm$ 6	86 $\pm$ 11	210 $\pm$ 18	307 $\pm$ 15
MAC 109	Water	41 $\pm$ 2	197 $\pm$ 34*	501 $\pm$ 39*	696 $\pm$ 38*
	7H9	43 $\pm$ 2	96 $\pm$ 14	256 $\pm$ 50	299 $\pm$ 10
MAC 104	Water	45 $\pm$ 2	152 $\pm$ 33*	387 $\pm$ 19*	665 $\pm$ 36*
	7H9	52 $\pm$ 10	82 $\pm$ 8	181 $\pm$ 26	273 $\pm$ 44
MAC A5	Water	42 $\pm$ 5	191 $\pm$ 24*	491 $\pm$ 30*	869 $\pm$ 30*
	7H9	43 $\pm$ 5	94 $\pm$ 14	243 $\pm$ 41	316 $\pm$ 17

*Statistically significantly different ($P < 0.05$) compared with biofilm formed in 7H9 medium at the same time-point.

Table 2. Effects of ions on *M. avium* biofilm formation

Strain MAC A5 was incubated in water with or without ions in a PVC 96-well plate and biofilm formation was measured as described in Methods. Values are means $\pm$ SD A_{570} readings from three experiments (multiplied by 1000).

Ion	Day 1	Day 7	Day 14
Water	50 $\pm$ 4	502 $\pm$ 74	933 $\pm$ 53
Mg ²⁺	0.1 μ M	50 $\pm$ 5	652 $\pm$ 13
	1 μ M	43 $\pm$ 8	731 $\pm$ 51*
	10 μ M	49 $\pm$ 3	789 $\pm$ 37*
			1632 $\pm$ 43*
Ca ²⁺	0.1 μ M	47 $\pm$ 6	632 $\pm$ 36
	1 μ M	53 $\pm$ 2	717 $\pm$ 47*
	10 μ M	46 $\pm$ 6	763 $\pm$ 24*
			1566 $\pm$ 37*
Fe ²⁺	0.1 μ M	53 $\pm$ 5	516 $\pm$ 30
	1 μ M	43 $\pm$ 7	531 $\pm$ 49
	10 μ M	47 $\pm$ 6	547 $\pm$ 28
			851 $\pm$ 54
Zn ²⁺	0.1 μ M	44 $\pm$ 3	561 $\pm$ 21
	1 μ M	48 $\pm$ 2	640 $\pm$ 37*
	10 μ M	51 $\pm$ 4	684 $\pm$ 24*
			1202 $\pm$ 27*

*Statistically significantly different ($P < 0.05$) compared with water control.

sources of carbon that may differ from the sources available in the host (lung airways, for example), we examined whether exposure to a number of sources of carbon would have an effect on the establishment of a biofilm. Table 3 shows that *M. avium* in water formed more stable biofilm in the presence of glucose and peptone, but not in the presence of humic

acid. Since different growth could explain the results obtained, we quantified *M. avium* on the three different media (three carbon sources), as described in Methods. No significant difference was observed in the number of bacteria (data not shown).

Analysis of GPLs of *M. avium* strains

A direct correlation has been made between the presence of GPLs, a class of amphiphilic molecules isolated in the outermost layers of the cell wall, and the ability of cells to translocate over a surface and the ability to form biofilm (Martinez *et al.*, 1999). To extend our analysis, we extracted the lipid from *M. avium* strains and performed TLC. Fig. 1 shows that MAC 101, MAC 104 and MAC A5 had significant amounts of polar GPL, while almost no polar GPL was detected in MAC 100. A semiquantitative analysis of the GPLs within the same culture was determined by integration of the iodine-stained GPLs using the NIH Image software (Table 4).

Effect of culture supernatant on biofilm formation

The ability of bacteria to form biofilms has been linked to quorum sensing, or the ability of micro-organisms to send messages to other members of the colony (Greenberg, 1998). Therefore, we decided to evaluate whether MAC A5 supernatant (7H9 broth of a culture) had any effect on biofilm formation in water. Table 5 shows that, in the presence of supernatant, there was a significant increase in the ability to form biofilm, compared with controls. Because the effect of supernatant suggests the presence of bacterium-derived molecules that affect biofilm formation, we placed MAC A5 in water in the presence of 4 μ g amikacin ml⁻¹, a subinhibitory concentration shown to inhibit protein synthesis (Bermudez *et al.*, 1997). Biofilm formation was reduced significantly (Table 5).

Table 3. Effect of carbon source on *M. avium* biofilm formation

Strain MAC A5 was incubated in the presence or absence of different carbon sources and biofilm formation was determined as described in Methods. Values are means $\pm$ SD A_{570} readings from three experiments (multiplied by 1000).

Carbon source	Day 1	Day 7	Day 14
Water (none)	34 $\pm$ 3	385 $\pm$ 46	714 $\pm$ 55
Glucose (2 %)	28 $\pm$ 6	853 $\pm$ 56*	1681 $\pm$ 104*
Peptone (1 %)	30 $\pm$ 4	1513 $\pm$ 177*	2310 $\pm$ 168*
Humic acid (0.05 mg ml ⁻¹)	29 $\pm$ 4	209 $\pm$ 27	667 $\pm$ 41

*Statistically significantly different ($P < 0.05$) compared with water control.

Fig. 1. TLC of GPLs isolated from *M. smegmatis* or *M. avium* strains MAC A5, MAC 5501, MAC 104, MAC 109, MAC 111 and MAC 100. GPLs were isolated from bacterial cultures as described in Methods. Results of a semiquantitative analysis of the GPLs are given in Table 4.

Resistance to biocids

Planktonic bacteria (10^7) and biofilm were incubated with biocids for 30 and 60 min, and the percentage of viable bacteria was then determined. As shown in Table 6, all the biocids were significantly more active against planktonic bacteria than against the biofilm form.

DISCUSSION

M. avium is a human pathogen that causes pulmonary

disease and disseminated infection (Inderlied *et al.*, 1993; Falkinham, 1996) in both immunosuppressed and immunocompetent individuals. *M. avium* is an environmental organism encountered in water and soil and, therefore, most of the human and animal population comes into contact with the bacterium during their lifetime. In the environment, *M. avium* can form biofilms, as studies have demonstrated (Carson *et al.*, 1978; Falkinham *et al.*, 2001; Martinez *et al.*, 1999; Schulze-Robbeke & Fischeder, 1989). The bacterium has been found in municipal water systems (Falkinham *et al.*, 2001) as well as on the walls of sauna houses in Finland (Schulze-Robbeke & Fischeder, 1989).

In this study, we observed that the ability of *M. avium* to form biofilm was strain-dependent under the conditions used. This was not surprising, given the heterogeneity of the *M. avium* strains. Among the strains tested, MAC 101, MAC 104, MAC 109 and MAC A5 are virulent in the mouse model, while MAC 100 is attenuated (Bermudez *et al.*, 1992). Based on this observation, it was tempting to correlate the ability to form biofilm with the virulence of the strain in the host, since MAC 100, the most attenuated strain in mice following intravenous or oral challenge, does not form film very effectively. However, we are not able to reach a definitive conclusion on this point. The results obtained in this study confirmed previous findings, that biofilm formation is more effective when bacteria are placed in water than in 7H9 broth medium (Limia *et al.*, 2001). Past observation has shown that, in water, but not in 7H9 broth, *M. avium* upregulates the expression of *secA*, which suggests that biofilm formation may be associated with protein secretion (Limia *et al.*, 2001).

Martinez *et al.* (1999) suggested that sliding motility was associated with the ability to form biofilm. We have confirmed this original observation (data not shown) and extended the finding to include a number of clinical isolates. Work by Martinez *et al.* (1999) also demonstrated that there was a close relationship between the capacity of a strain for sliding motility and biofilm formation and the amount of GPL. GPL is believed to form a hydrophobic film over the hydrophilic agar surface, reducing interaction between the bacterium and the agar and allowing spreading. Although this corresponds to a phenotype *in vitro*, the relevance of sliding motility in the environment is currently unknown. It is possible that GPLs are not the only bacterial component responsible for sliding motility, since *M. avium* and *M. smegmatis* GPLs show significant variations, while both have sliding motility on agar.

The quality and quantity of nutrients, ions and several other substances present in the environment are known to influence bacterial behaviour and would certainly be expected to have a regulatory role in the formation of biofilm. For example, *Pseudomonas aeruginosa* uses organic acids as a source of carbon to form biofilm (O'Toole *et al.*, 2000). In addition, Kirschner *et al.* (1992) showed that *M. avium* grows better in water rich in zinc and humic acid. Our work demonstrates that *M. avium* establishes more stable biofilms in the presence of Ca^{2+} , Mg^{2+} and Zn^{2+} , but not Fe^{2+} . In

Table 4. Semiquantitative analysis of GPLs

Iodine-stained GPL bands (numbered as shown in Fig. 1) were integrated using the NIH Image software. Values are percentages of total integrated densities of the desired GPLs within a single lane. NI, Not included.

Band	R_f	<i>M. avium</i>						<i>M. smegmatis</i>
		MAC A5	MAC 5501	MAC 104	MAC 109	MAC 101	MAC 100	
1	0.14	5.88	6.29	12.82	0.58	10.86	7.98	0.38
2	0.21	2.33	19.42	48.94	4.49	4.75	10.03	4.44
3	0.23	0.00	0.00	0.00	0.00	1.27	77.49	0.05
4	0.35	2.42	0.84	0.00	0.00	0.00	0.25	0.13
5	0.48	32.59	27.47	1.52	14.13	0.00	0.00	2.89
6	0.56	6.17	3.01	0.72	9.18	6.56	0.00	43.07
7	0.69	37.43	26.95	7.16	43.25	23.45	0.00	9.72
8	0.78	11.03	6.61	5.08	10.12	17.07	1.05	39.31
9	0.85	2.14	9.41	23.76	18.26	36.03	3.20	NI

Table 5. Effect of culture supernatant and inhibition of protein synthesis on *M. avium* biofilm formation

Culture supernatant was obtained as described in Methods. Strain MAC A5 was incubated in the media shown and biofilm formation was determined as outlined in Methods. Values are means $\pm$ SD A_{570} readings from three experiments (multiplied by 1000).

Medium	Day 1	Day 7
Water	56 $\pm$ 8	426 $\pm$ 12
Water+supernatant (50 μ l)	52 $\pm$ 4	673 $\pm$ 46*
Water+7H9 (50 μ l)	50 $\pm$ 2	301 $\pm$ 26
Water+amikacin (4 μ g ml ⁻¹)†	47 $\pm$ 4	186 $\pm$ 11*
7H9 (50 μ l)	44 $\pm$ 6	346 $\pm$ 15

*Statistically significantly different ($P < 0.05$) compared with water control.

†MIC of amikacin for strain MAC A5 is 12 μ g ml⁻¹.

addition, glucose and peptone were sources of carbon in medium associated with increased biofilm formation. Humic acid, however, was associated with less biofilm formation. The differences between our results and those of Kirschner *et al.* (1992) might result from the strains used or the contents of the medium. It is possible that, because humic acid chelates cations, including Ca²⁺ and Mg²⁺, it decreased the number of divalent cations available for efficient biofilm formation. Since *M. avium* has been shown to establish biofilms in the water systems of urban areas, as well as on the walls of public saunas, one wonders whether those conditions linked with biofilms *in vitro* are present in the environments in which *M. avium* biofilms have been identified. Of note is the fact that increased amounts of zinc have been found in urban water systems (Kirschner *et al.*, 1992). In addition, we believe that other sources of carbon besides those tested can be associated with *M. avium* biofilm formation. As an extension of this work, it would be interesting to investigate the conditions that exist in the

Table 6. Bactericidal effect of biocids against *M. avium*

Inocula of approx. 10⁷ planktonic bacteria and bacteria in biofilm (sessile) were incubated with several biocids. At the times shown, the biocid was removed by washing and the bacterial load was determined by plating into 7H100 agar plates. Values are means $\pm$ SD c.f.u. ml⁻¹.

Biocid	Form	30 min	1 h
None	Planktonic	1 $\pm$ 0.4 $\times$ 10 ⁷	1.3 $\pm$ 0.2 $\times$ 10 ⁷
	Biofilm	3.2 $\pm$ 0.5 $\times$ 10 ⁸	3.3 $\pm$ 0.4 $\times$ 10 ⁸
Sodium hypochloride (0.5 %)	Planktonic	8.1 $\pm$ 0.5 $\times$ 10 ⁶	7.4 $\pm$ 0.4 $\times$ 10 ⁶
	Biofilm	2.9 $\pm$ 0.4 $\times$ 10 ⁷	1.9 $\pm$ 0.3 $\times$ 10 ⁷
Potassium monopersulfate (21 %)	Planktonic	2.4 $\pm$ 0.2 $\times$ 10 ⁶	5.1 $\pm$ 0.5 $\times$ 10 ⁵
	Biofilm	8.7 $\pm$ 0.3 $\times$ 10 ⁶ *	9.3 $\pm$ 0.2 $\times$ 10 ⁶ *
Chloroheximide acetate (2 %)	Planktonic	7.9 $\pm$ 0.4 $\times$ 10 ⁵	8.3 $\pm$ 0.6 $\times$ 10 ⁴
	Biofilm	6.9 $\pm$ 0.4 $\times$ 10 ⁶ *	7.7 $\pm$ 0.3 $\times$ 10 ⁶ *

*Statistically significantly different ($P < 0.05$) compared with the effect on planktonic bacteria.

airways of patients with bronchiectasis, for instance, and to establish whether those conditions could facilitate biofilm formation by *M. avium* strains.

A connection between biofilm formation and quorum sensing has been established for a number of bacteria (Greenberg, 1998). Our results suggest that similar mechanisms may have a role in the formation of *M. avium* biofilm. The facts that culture supernatant led to a significant increase in the ability to establish biofilm and that inhibition of protein synthesis suppressed biofilm formation potentially indicate that a protein-mediated signal(s) present in the supernatant stimulates biofilm formation.

The role of biofilm in *M. avium* pathogenesis is unknown. Because *M. avium* colonizes the respiratory tract of patients with chronic pulmonary disease and the initial colonization usually evolves to an infection that is difficult to eradicate, it is plausible that biofilm formation might have a substantial role in the maintenance of the mucosal infection. The observation that *M. avium* in biofilm form is significantly more protected from the action of biocids than are bacteria in planktonic form supports the idea that the phenotype is adopted for protection against the harmful conditions of the environment. Further work is necessary to establish this association.

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