

Melanin synthesis in microorganisms – biotechnological and medical aspects*

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Melanins form a diverse group of pigments synthesized in living organisms in the course of hydroxylation and polymerization of organic compounds. Melanin production is observed in all large taxa from both Pro- and Eukaryota. The basic functions of melanins are still a matter of controversy and speculation, even though their adaptative importance has been proved. Melanogenesis has probably evolved paralelly in various groups of free living organisms to provide protection from environmental stress conditions, but in pathogenic microorganisms it correlates with an increased virulence. The genes responsible for melanization are collected in some cases within operons which find a versatile application in genetic engineering. This review summarizes current views on melanogenesis in Pro- and Eukaryotic microorganisms in terms of their biotechnological and biomedical importance.

Keywords: melanogenesis, bacteria, fungi, protozoa, myxomycetes, virulence

INTRODUCTION

Production of melanin is one of the most universal, but at the same time enigmatic adaptations of living organisms to the variable conditions of the Earth. The presence of various kinds of melanins in representatives of almost every large taxon suggests an evolutionary importance of melanogenesis. Up to now, no agreement has been reached concerning the primary or basic function of this process and its product, or even concerning its character as a primary or side effect. Just the opposite – it is the matter of a permanent discussion and argument (Wood *et al.*, 1999). Numerous reviews are available on the biology, genetics, and medical implications of melanogenesis in higher animals and in human (Zecca *et al.*, 2001; Halaban, 2002; Slominski *et al.*, 2005). Much less is known about melanogenic pathways in microorganisms. Practical implications of these phe-

nomena prompted us to focus on this topic and to present at least the most important examples. This may shed new light on the evolution of melanogenesis in higher organisms as well.

Melanins are polymers of phenolic compounds. The more general classification of such compounds, including all their types in Pro- and Eukaryota, contains three main types of such polymers:

Eumelanins (black or brown) – produced in the course of oxidation of tyrosine (and/or phenylalanine) to *o*-dihydroxyphenylalanine (DOPA) and dopaquinone (Fig. 1), which further undergoes cyclization to 5,6-dihydroxyindole (DHI) or 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (del Marmol & Beermann, 1996; Langfelder *et al.*, 2003);

Pheomelanins (yellow-red) – which are initially synthesized just like eumelanins, but DOPA undergoes cysteinylolation, directly or by the media-

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Abbreviations: DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; DHN, dihydroxynaphthalene; DOPA, 3,4-dihydroxyphenylalanine; HPA, 4-hydroxyphenylacetic acid hydroxylase; HPPD, hydroxyphenylpyruvate dehydrogenase; HPPH, hydroxyphenylpyruvate hydroxylase; LD, Legionnaire's disease; LPS, lipopolysaccharide; PKS, polyketide synthase; RNS, reactive nitrogen species; ROS, reactive oxygen species; SDS, sodium dodecyl sulphate; SOD, superoxide dismutase; Tat, twin-arginine translocation.

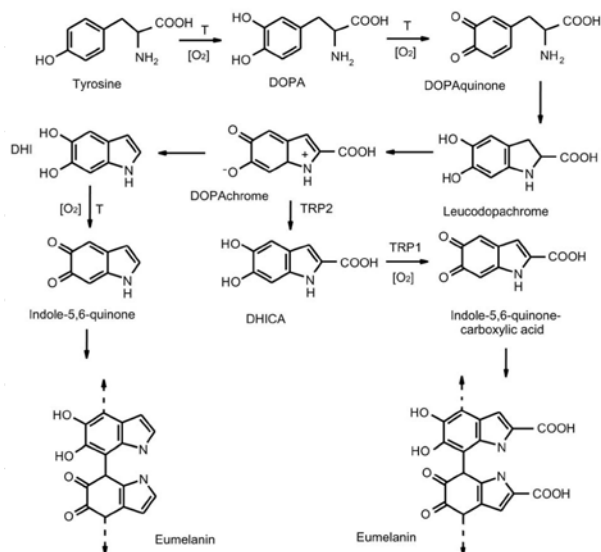


Figure 1. Eumelanogenesis pathway.

T, tyrosinase; TRP1, tyrosinase related protein 1; TRP2, tyrosinase related protein 2; DOPA, 3,4-dihydroxyphenylalanine; DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid (after Nappi & Ottaviani, 2000; Kobayashi *et al.*, 1995; modified).

tion of glutathione (Fig. 2). The end product of this reaction, cysteinylDOPA, further polymerizes into various derivatives of benzothiazines (Kobayashi *et al.*, 1995; Nappi & Ottaviani, 2000);

Allomelanins — the least studied and the most heterogeneous group of polymers, which emerge through oxidation/polymerization of di- (DHN) or tetrahydroxynaphthalene, *via* the pentaketide pathway leading through flavioline to various coloured polymers of DHN-melanins (Fig. 3A), homogentisic acid (pyromelanins) (Fig. 3B), γ -glutaminy-4-hydroxybenzene, catechols, as well as of 4-hydroxyphenylacetic acid (Gibello *et al.*, 1995; Kotob *et al.*, 1995; Espin *et al.*, 1999; Funa *et al.*, 1999; Jacobson, 2000).

In general, pheomelanins contain sulphur, whereas most types of allomelanins do not contain nitrogen. The second important difference concerns configuration of quinone or quinonimine residues in melanins: *ortho*- configuration is present in eu- and

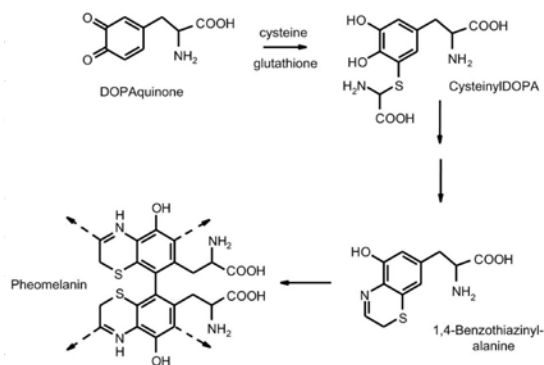


Figure 2. Pheomelanin biosynthesis pathway after (Nappi & Ottaviani, 2000; Kobayashi *et al.*, 1995, modified).

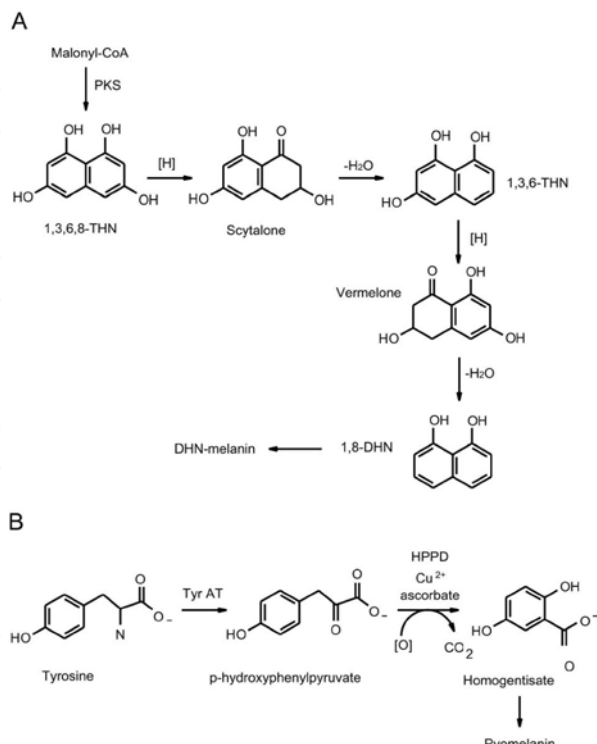


Figure 3. Examples of allomelanin biosynthesis pathways.

A, synthesis of DHN melanin. PKS, polyketide synthase; 1,3,6,8-THN, 1,3,6,8-tetrahydroxynaphthalene; 1,3,6-THN, 1,3,6-trihydroxynaphthalene; 1,8-DHN, 1,8-dihydroxynaphthalene (after Langfelder *et al.*, 2003; Funa *et al.*, 1999; modified). B, synthesis of pyromelanin from tyrosine. Tyr AT, tyrosyl aminotransferase; HPPD, hydroxyphenylpyruvate dehydrogenase.

pheomelanins, and *para*- (polymers of γ -glutaminy-4-hydroxybenzene) or *meta*- (polymers of DHN) in allomelanins.

ENZYMES OF MICROBIAL MELANOGENESIS IN RELATION TO THEIR ANIMAL COUNTERPARTS

Tyrosinases (EC 1.14.18.1), which catalyze oxidation of monophenols to *o*-dihydroxyphenols and further to *o*-quinones, have a putative common ancestor with oxygen-binding and oxygen-transporting proteins (Dursewitz & Terwilliger, 1997; Decker & Terwilliger, 2000). They appeared on the Earth in consequence of the change in the chemical character of the atmosphere from reducing to oxidizing one, which was a result of photosynthesis. They originate from primitive metalloproteins, which bind dioxygen through the prosthetic group that contains a coordinated transient metal cation.

The hemocyanin-like proteins family comprises molluscan and arthropod hemocyanins, tyrosinases, prophenoloxidases, and insect hexamerins (Burmester & Scheller, 1996). All of them reveal a conserved position of Cu-coordinating histidine residues which form the CuA and CuB domains (Bur-

mester & Scheller, 1996). Only the latter one is conserved in all taxa. The CuA domain from arthropod hemocyanin differs greatly from the molluscan counterpart, which is similar to the CuA domain present in various tyrosinases (including Prokaryotic ones). This is a possible consequence of evolutionary parallelism (Lieb *et al.*, 2001). Arthropod hemocyanins form a monophyletic group with prophenoloxidases. These two families of proteins are closely related, whereas the homology to molluscan hemocyanins and tyrosinases from other animal taxa, which form a sister group, remains distant (Fujimoto *et al.*, 1995). It is accepted that the structure of the CuB domain is ancient and was established very early in evolution. It is particularly characteristic for tyrosinases, which are present in both Pro- and Eukaryota: a homology between *Streptomyces* sp. and mouse tyrosinases can still be noticed (Lerch, 1988). Molluscan hemocyanins and tyrosinases most probably originate from a single polypeptide forming two domains — CuA and CuB, while arthropod hemocyanins are the product of a duplication and subsequent fusion of CuB-encoding genes (Kawabata *et al.*, 1995; Immesberger & Burmester, 2004). The unique common feature of molluscan hemocyanins and fungal tyrosinases is the thioether bridge between cysteine and histidine residues conservatively located in the CuA domain (Lerch, 1983; 1988; van Gelder *et al.*, 1997). The similarity of molluscan hemocyanins and tyrosinases is also manifested by their ability to oxidize *o*-diphenols to *o*-quinones under physiological conditions. Conversely, with arthropod hemocyanins it can be achieved only after a limited proteolysis or SDS denaturation (Decker & Rimke, 1998; Salvato *et al.*, 1998; Decker *et al.*, 2001).

Laccases (oxidoreductases *p*-diphenol:dioxygen, EC 1.10.3.2) are also metalloproteins containing one to four copper atoms in their active site (Valderrama *et al.*, 2003). Not related to tyrosinases, they belong to the family of blue copper-containing oxidases, together with ascorbate oxidase and ceruloplasmin. They form a strongly diversified group of low homology, and very little is known about their evolution — much less than in the case of tyrosinases. Both types of these melanogenic enzymes can be found in Eukaryota and in Prokaryota, which confirms their ancient evolutionary origin (Messerschmidt & Huber, 1990; Valderrama *et al.*, 2003).

Polyketide synthases (PKS) produce DHN melanins, and belong to an old family of multidomain proteins related to the animal fatty acid synthases (Kroken *et al.*, 2003). The PKS family is diversified and plentiful. Numerous microorganisms employ these enzymes to produce pigments, antibiotics, toxins and other products of intermediate metabolism (Hutchinson, 2003; Snyder *et al.*, 2003). There are only few DHN-melanin-producing PKS

enzymes, which belong to the PKS-type-I group producing aromatic, not reduced polyketides (Kroken *et al.*, 2003).

Some species of bacteria produce and secrete pyomelanin, a product of homogentisic acid polymerization. One of the main enzymes engaged in pyomelanin production is *p*-hydroxyphenylpyruvate oxidase (HPPD, also known as *p*-hydroxyphenylpyruvate hydroxylase — HPPH; EC 1.13.11.27), which catalyses homogentisinic acid (alkapton) formation (Kotob *et al.*, 1995). This enzyme, which belongs to the phenylalanine and tyrosine degradation pathway, is ubiquitous among living organisms. Deregulation of this metabolic pathway may lead to the retention of potentially toxic alkapton in the cells and tissues (Menon *et al.*, 1991; Phornphutkul *et al.*, 2002).

Another melanogenic enzyme, **4-hydroxyphenylacetic acid hydroxylase** (HPA, EC 1.14.13.3), belongs to a separate family of hydroxylases (Gibello *et al.*, 1997). Besides its main substrate, 4-phenylacetic acid it catalyzes hydroxylation of other aromatic compounds, which leads to the formation of dibenzoquinone and other *o*-quinone derivatives, which then polymerize spontaneously to allomelanin-like polymers (Gibello *et al.*, 1995). Tyrosine is also a substrate of HPA, but (unlike tyrosinase) this enzyme does not contain copper, which does not increase its enzymatic activity.

MELANINS AS ELECTRON ACCEPTORS IN DISSIMILATING BACTERIA

In the process of anaerobic respiration, reducing, dissimilating bacteria use a wide spectrum of electron acceptors replacing dioxygen in the last step of the respiratory chain. Among the substances utilized as alternative electron acceptors, there are mainly hydrated ferrous oxide, nitrates, sulphates, as well as organic compounds, in particular the ones containing quinone groups, e.g. related to melanin humic substances (Coates *et al.*, 2002).

Melanin, similarly to humic substances, is a polymer of various groups able to donate or to accept an electron. Therefore it can act as a final acceptor or a shuttle in the electron exchange with insoluble compounds of iron (Menter & Willis, 1997). The facultatively anaerobic bacterium *Shewanella algae* produces pyomelanin and reduces it simultaneously with the oxidation of gaseous hydrogen (Turick *et al.*, 2002). Having accepted numerous electrons, such “reduced” melanin serves the bacteria as a reductor of insoluble ferric (III) oxides to the ferrous (II) state (Fig. 4). As *S. algae* is unable to carry out fermentation, its survival in the conditions of variable oxygen concentration strongly depends on the presence of

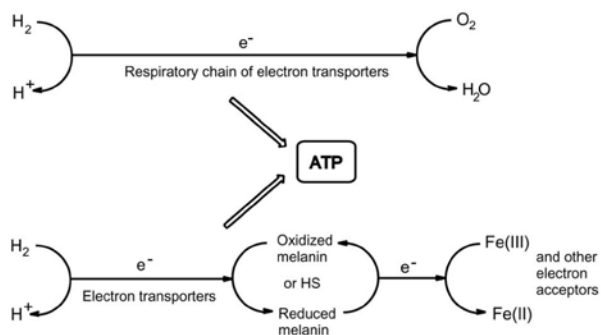


Figure 4. Possible function of melanin as an electron acceptor in the respiratory chain of marine bacteria, in comparison to the aerobic variant of the process.

HS, humic substances.

appropriate electron acceptors (Turick *et al.*, 2002). In the mineralized marine deposits the availability of such soluble compounds is limited, therefore production of melanin is an important evolutionary adaptation. Moreover, like other organisms, *S. alga* produces melanin also for the protection from ultraviolet irradiation.

A similar, still poorly understood phenomenon concerns another marine bacterium — *Marinomonas mediterranea*, which has been discovered to contain two melanin-generating enzymes, namely tyrosinase and laccase with a wide spectrum of activity (Sanchez-Amat *et al.*, 2001; Lopez-Serrano *et al.*, 2002). As it was shown by mutation analysis, only tyrosinase is indispensable for the production of the dark pigment and laccase has other physiological functions (Sanchez-Amat *et al.*, 2001; Lopez-Serrano *et al.*, 2002). The co-expression of both enzymes is unique to Prokaryota. The natural substrate for *M. mediterranea* melanogenesis in the oligotrophic sea water remains unknown. The existence of microenvironments rich in organic compounds cannot be excluded. An example of such a niche are surfaces biofilms, where concentration of amino acids may reach 10–30 nM (Lucas-Elio *et al.*, 2002). Some aromatic compounds and polyphenols can be oxidized both by tyrosinase and by laccase. Many species of Fungi employ laccase to decompose lignins and other organic compounds (Leonowicz *et al.*, 2001). Therefore another function of laccase, evolutionarily beneficial to marine bacteria, would be decomposition of deposits of poorly accessible organic matter.

MELANIN IN ATMOSPHERIC NITROGEN FIXATION BY BACTERIA

The soil aerobic bacterium *Azotobacter chroococcum* contains an active polyphenol oxidase and can produce melanin from catechol (Shivprasad & Page, 1989). This microorganism produces particu-

larly large amounts of melanin when cultured under aerobic conditions. This is easy to explain, as oxygen is one of the substrates for polyphenol oxidase, but it turns out that this process is intensified in the absence of a nitrogen source in the medium (Shivprasad & Page, 1989). Although the intensity of melanogenesis does not seem to be directly correlated with the activity of nitrogenase (the key enzyme of atmospheric nitrogen fixation), it is possible that *Azotobacter* employs melanogenesis to enhance utilization of oxygen and to maintain reducing conditions necessary for binding atmospheric nitrogen. The presence of iron and copper ions in the medium significantly increases the rate of *Azotobacter* melanization. This is not only because polyphenol oxidase requires the presence of Cu ions in the active site. Enhanced melanogenesis also confers a protective role against hydroxyl radicals (OH \cdot) produced in the Fenton reaction which takes place in the presence of Cu and Fe ions (Shivprasad & Page, 1989; Sutton & Winterbourn, 1989). This protection involves providing catechol and binding metal ions in the structure of melanin. Catechol, an intermediate in the melanogenic pathway, is a potential target of the oxidizing hydroxyl radical, and a potential “sweeper” of this extraordinarily active form of oxygen (Nasr *et al.*, 2005).

In the symbiotic *Rhizobium* bacteria living in the root nodules of *Papilionaceae* plants, genes encoding melanogenic enzymes, including tyrosinase, are located in plasmids required for the development of the symbiosis (Hawkins & Johnston, 1988). Surprisingly, these genes do not seem to be obligatory for the development of the nodules or for the nitrogen fixation itself. The function of these genes in *Rhizobium* is still unclear, but one of postulated explanations suggests that tyrosinase may play a role in detoxication by oxidation of phenolic compounds deposited in ageing root nodules (Hynes *et al.*, 1988).

MELANIN AS A FACTOR OF VIRULENCE IN PATHOGENIC BACTERIA

The ability of pathogenic bacteria to produce melanin seems to originate from the evolutionary achievements of free-living bacteria. In some genera containing both free-living and parasitic strains (e.g. *Vibrio* sp.), there are both pyromelanogenic and eu/pheomelanogenic pathways, sometimes simultaneously active in one organism. Free-living strains of *Vibrio cholerae* are usually amelanotic or they produce pyromelanin (Kotob *et al.*, 1995). Meanwhile, under stress (hyperthermia, hyperosmotic medium, starvation) they induce synthesis of eumelanin, and a pathogenic mutant HTX-3 — ph-

eomelanin (Ivins & Holmes, 1981; 1980; Coyne & Al-Harthy, 1992).

Production of pyomelanin has been reported in many species of bacteria, like *Pseudomonas aeruginosa*, *Hypomonas* sp., *Shewanella colwelliana*, some of which consist of both free-living (marine) and pathogenic strains (Shivprasad & Page, 1989). *Legionella pneumophila* may serve as another example of the latter group. It causes the so called Legionnaire's disease (LD) and Pontiac fever (Wintermeyer *et al.*, 1994; Rathore & Alvarez, 2006). One of the factors responsible for the pathogenicity of this microorganism is legiolysin (Lly), a protein responsible for the fluorescent properties, pigment production and hemolytic ability of the pathogen (Wintermeyer *et al.*, 1991). It reveals over 80% similarity with HPPD (the product of *MelA* gene — see later), crucial for melanin production in *Schewanella colwelliana* and in *Pseudomonas* sp. (Fuqua *et al.*, 1991; Fuqua & Weiner, 1993; Wintermeyer *et al.*, 1994; Steinert *et al.*, 2001). This key enzyme for pyomelanogenesis confers hemolytic activity as well (Croxatto *et al.*, 2002). The expression of HPPD is regulated in a similar way to other genes responsible for the virulence factors, such as production of metalloproteinases and extracellular lipopolysaccharides, used to create biofilms and necessary for adhesion to biological surfaces like fish skin (Croxatto *et al.*, 2002).

The pathogenic bacterium *Burkholderia cepacia* serves as an example of how melanin production increases virulence. This microorganism causes dangerous lung infections, which often develop to sepsis, mainly due to the presence of lipopolysaccharide (LPS) which strongly enhances production and release of proinflammatory cytokines (Zughaier *et al.*, 1999). Although LPS does not trigger an oxygen burst directly, it stimulates the immunological system to an accelerated oxidative response to other stimuli. Melanin isolated from *B. cepacia* reveals a dose-dependent ability to sweep O_2^- produced by leukocytes during oxygen burst. However, melanin does not influence the release or kinetics of the production of reactive oxygen species (ROS) (Zughaier *et al.*, 1999). The ability to remove superoxide anion allows *B. cepacia* to survive phagocytosis, so the host phagocytes are not able to eliminate the pathogen. They remain in the state of a permanent stimulation by the bacterial LPS, which causes a chronic inflammation. Similarly to *Legionella pneumoniae*, *B. cepacia* is able not only to survive in the phagocytes (alveolar macrophages), but also to proliferate intracellularly, which leads to cell destruction and to the infection of secondary macrophages (Saini *et al.*, 1999; Abu-Zant *et al.*, 2005).

In *Proteus mirabilis*, the second (after *E. coli*) important cause of community-acquired infections of the urinary tract, tyrosinase was identified as

the enzyme responsible for melanization. As it has been shown in this species, melanin decreases the level of ROS, which probably makes the pathogen more resistant to the oxygen burst connected with the immunological response of the host (Agodi *et al.*, 1996).

Klebsiella pneumoniae, responsible for a particularly dangerous form of pneumonia, produces melanin from 4-hydroxyphenylacetic acid using HPA, which is an unusual pathway of melanogenesis (Gibello *et al.*, 1995; 1997). However, the importance of HPA and melanin for the virulence of this pathogen remains to be determined.

In a separate study, the sequence of fungal laccase was used to search for homologous genes in Prokaryota (Alexandre & Zhulin, 2000). Similar sequences were found in 14 bacterial species, including such important human pathogens as *E. coli*, *Bordetella pertussis*, *P. aeruginosa*, *Campylobacter jejuni*, *Yersinia pestis*, or mycobacteria, including *Mycobacterium leprae*. In all these pathogens the production of melanin or laccase activity should be suspected as a potential mechanism of virulence. *M. leprae* has an ability, unique among mycobacteria, to oxidize diphenols to *o*-quinones, and in consequence oxidation of L-DOPA has become a convenient diagnostic feature for *M. leprae* (Prabhakaran *et al.*, 1977; Prabhakaran & Harris, 1985).

MOLECULAR GENETICS OF BACTERIAL MELANOGENESIS

The genetic background of melanogenesis in prokaryotic microorganisms has been elucidated only in several cases, even though a large number of examples of melanin production have been described.

In the case of *Streptomyces*, the ability to produce pigment is an important taxonomical criterium. In *Streptomyces antibioticus* the *melC* operon controls eumelanin production (Chen *et al.*, 1992). It consists of two structural genes in the extrachromosomal part of the genome. The first open reading frame (ORF438, *melC1*, 438 bp) encodes MelC1 protein (M_r 14 754), and the second gene (ORF816, *melC2*, 816 bp) encodes apotyrosinase (M_r 29 500) (Bernan *et al.*, 1985). The main factor inducing expression of *melC* in *S. antibioticus* is L-methionine (Katz & Betancourt, 1988).

The actual role of MelC1 has been revealed only recently. First of all, it has been shown that this protein forms a heterodimer with tyrosinase, acting as its *trans*-activator (Chen *et al.*, 1993). Next, it was demonstrated that this interaction is necessary to incorporate two copper ions crucial for the enzymatic activity of the enzyme. Incubation of apotyrosinase

with copper ions did not lead to the full restoration of enzymatic activity, if it was not supplemented with MelC1. This protein seems, therefore, to possess a chaperone-like function (Chen *et al.*, 1992). Even more interestingly, it is not only responsible for the proper folding and incorporation (proper folding by incorporation?) of the metal ions into the active site, but also enables the secretion of native tyrosinase by *S. antibioticus* (Leu *et al.*, 1992). Tyrosinase is secreted in complex with MelC1. Recently, a deeper biological sense of this phenomenon has been discovered. The complex is secreted using one of the three known bacterial mechanisms of protein secretion, namely – *via* interaction with Tat (Twin-arginine translocation) proteins (Schaerlaekens *et al.*, 2001). The mechanism of their action is related structurally and functionally to the mechanism generating the pH gradient in thylakoids of chloroplasts. It turned out that only MelC1 contains the signal peptide responsible for translocation of the dimer. This is the reason for which tyrosinase is secreted only when bound to the MelC1 protein, as a ready, natively-folded protein. This mechanism (the so-called “hitchhiker” mechanism) based on the interactions with Tat proteins is also responsible for the secretion of the product of a two-gene operon of hydrogenase 2 in *E. coli*. However, the primary biological meaning of the presence of tyrosinase in the secretomes of some *Streptomyces* species remains unknown.

The genome of *Marinomonas mediterranea* also contains a two-cistron operon, *ppoB*, responsible for melanogenesis (Lopez-Serrano *et al.*, 2004). The *ppoB1* gene encodes apotyrosinase and *ppoB2* a chaperone incorporating copper ions to the active site of the enzyme. While there are known orthologues of *ppoB1* in *Eucaryota* and some bacteria, including *Streptomyces* sp., the *ppoB2* product does not reveal any significant homology either with MelC1 of *Streptomyces antibioticus*, or with other proteins checked in this context. Interestingly, PpoB2 contains six transmembrane helices and is not secreted, unlike its functional counterpart in streptomycetes (Lopez-Serrano *et al.*, 2004).

M. mediterranea contains also another melanogenetic enzyme – laccase (*ppoA* gene) (Sanchez-Amat *et al.*, 2001). Regulation of *ppoA* and *ppoB* expression proves the evolutionary importance and strongly adaptative character of melanin production for this species. It is regulated *via* a two-component system of signal transduction, which consists of a membrane-anchored sensor histidinyI kinase and its intracellular response regulator (Lucas-Elio *et al.*, 2002). After receiving an environmental stimulus, the extracellular, N-terminal domain of the kinase undergoes a conformational transformation leading to the autophosphorylation of the histidine residues in the C-terminal, intracellular domain of the pro-

tein. The phosphate group is then transferred onto an aspartyl residue of the response-regulating intracellular protein, which finally leads to the cellular response. The *M. mediterranea* sensor kinase (PpoS – polyphenol oxidase sensor kinase) has a structure similar to the BvgS kinase regulating the virulence of *Bordetella pertusis* (Lucas-Elio *et al.*, 2002). It has been shown that a mutation of *ppoS* down-regulates the expression of tyrosinase and laccase in bacteria under stress conditions, including, e.g., the stationary growth phase when the resources of the medium begin to dwindle.

In free-living *Shewanella colwelliana* with Mel+ phenotype, a monocistronic, 1.3 kb *melA* operon has been identified. It contains an ORF with the transcription initiation site 115 nucleotides upstream of the putative translation initiation site. The *melA*-encoded protein (39.5 kDa, 346 amino acids) was identified as HPPD found also in *Vibrio cholerae*, *Hypomonas* sp., or *Pseudomonas* sp. (Fuqua *et al.*, 1991).

The *Klebsiella pneumoniae* 4-phenylacetic acid hydroxylase activity is determined by two proteins encoded by separate cistrons – *hpaA* and *hpaH*. Their expression in *E. coli* resulted in production of a brown pigment in the medium. *hpaA* encodes a flavoprotein (58781 Da) with the enzymatic activity, while the *hpaH* product (18680 Da) is a “helper” protein, necessary to achieve a high catalytic efficiency of HPA (Gibello *et al.*, 1997).

BACTERIAL MELANOGENESIS IN BIOTECHNOLOGY AND GENETIC ENGINEERING

The enzymatic activity of tyrosinase is manifested in bacteria by extracellular melanin production, and by easily noticeable darkening of the medium. Therefore it is easy to quantify the expression of a tyrosinase gene or the whole *melC* operon, e.g. by a spectrophotometric assay of the medium. This phenomenon has been widely employed in genetic engineering, and the *melC* operon or its constituents (the structural part devoid of the promoter or *melC2* alone) are parts of various gene constructs serving as reporter genes (Paget *et al.*, 1994). A successful transfer of *melC* genes of *S. antibioticus* has been reported for various expression systems, including other streptomycetes (e.g. *S. lividans*) and *E. coli* (Katz *et al.*, 1983; della Cioppa *et al.*, 1990; Tseng *et al.*, 1990). A remarkable example of the utilization of this operon in molecular investigations concerns the studies on transcription factors of the highly pathogenic *Streptococcus thermophilus*. Particularly promising are the results of a study on the structural genes of the *melC* operon from *Streptomyces antibioticus* under the control of streptococcal promoters expressed in *E. coli* (Solaiman & Somkuti, 1995; 1997). Even

more spectacular is the successful transfection of amelanotic murine skin cells with *melC*, leading to the restoration of the pigmented phenotype of the murine cells (Zhao *et al.*, 2000). Such experimental approach may in future lead to new methods of investigation of animal melanogenesis, but also to new therapeutic strategies against skin and hair pigmentation disorders.

An opposite approach, where the expected effect was the lack of melanin synthesis, was used in the construction of combinatorial libraries of cyclic peptides and proteins (Scott *et al.*, 1999). Peptides encode five times more information than nucleic acids of the same number of mers. The maintenance of a satisfactory level of stable products which easily undergo catabolic decomposition is highly problematic. One of the solutions is cyclization of peptides and oligopeptides using inteins — fragments of polypeptides or proteins appearing as intermediate conformers and revealing their own catalytic activity. Such an activity is transient, and is usually connected with a specific excision and/or ligation of other fragments of oligopeptide chain during post-translational modification. The authors used an inhibitor of tyrosinase — pseudostellarin F, a cyclic octapeptide, as a product of intein activity. The proof for the presence and activity of the whole gene construct, i.e. for the actual appearance of the intein and its expected ligase activity is the lack of tyrosinase activity, manifested by the lack of medium darkening. Such a system may be used to stabilize other oligopeptides, besides pseudostellarin F. It should be noticed that the intein system originated from a cyanophyta — *Synechocystis* sp. (PCC6803 Ssp), the tyrosinase and *melC1* gene from *Streptomyces antibioticus*, and the library was expressed in *E. coli*.

Besides pseudostellarin F, bacteria are also a source of other specific, efficient and often nontoxic inhibitors of tyrosinase, or of melanogenesis in general. Such inhibitors have been isolated e.g. from the culture medium of *Streptomyces* sp. or *S. clavifer* (Ishihara *et al.*, 1991; Komiyama *et al.*, 1993; Lee *et al.*, 1997). Finally — it must be mentioned that various authors suggest the possibility to employ bacteria directly to produce various kinds of melanins for further use in industry (della Cioppa *et al.*, 1990).

PROTOZOA

This group is probably the least explored one in respect to melanogenesis and melanin function among eukaryotic organisms. Melanin is usually noticed in the context of the response of invertebrates to microbial parasites, e.g. in the case of mosquitoes (*Anopheles*) being invaded by *Plasmodium* (Chun *et al.*, 2000; Cui *et al.*, 2000; Kumar *et al.*, 2003; Volz *et*

al., 2006). The host usually reacts with encapsulation of the parasite and melanization of the surrounding tissues. This basic type of invertebral defense depends in mosquitoes on melanization-regulating genetic module or network encompassing at least 14 genes (Volz *et al.*, 2006). However, melanin is here produced by the host, not by the protozoal parasite (Nappi & Ottaviani, 2000; Kumar *et al.*, 2003).

Many protozoal species are able to synthesize melanin precursors which may spontaneously polymerize to form the pigment. An interesting example is the “pig” (which stands for “pigmented”) mutant of *Tetrahymena thermophila*, able to secrete huge amounts of allomelanin precursors to the culture medium (Kaney & Knox, 1980; Martin Gonzalez *et al.*, 1997). These compounds, mostly products of catecholamine oxidation, spontaneously polymerize to melanin, which is likely to be a result of hyperactivity of aromatic amino-acids decarboxylase (L-DOPA decarboxylase, EC 4.1.1.26). Activity of this enzyme, as in the case of bacteria, can be easily observed and quantified in the culture. Therefore, the “pig” mutant has been employed to test the effectiveness of some eukaryotic antibiotics and mycotoxins (Martin Gonzalez *et al.*, 1997).

The marine unicellular algae *Dinoflagellata* have been shown to express their own PKS-type-I enzymes encoded by dinoflagellate-resident genes (Snyder *et al.*, 2005) responsible for the production of various neurotoxins and potential anti-cancer agents (Rein & Borrone, 1999; Snyder *et al.*, 2003), but also substances toxic for fish (Berry *et al.*, 2002). Although melanin synthesis in these organisms has not been reported yet, this phenomenon seems to be worth noticing for several reasons — it concerns a potentially melanogenic pathway, and serves as another example of the existence of such pathways in *Protista*, it documents a quite unusual localization and employment of PKS, and finally, it has clear practical (medical and biotechnological) aspects.

FUNGI

Melanin biosynthesis is a common feature in the kingdom of Fungi, but for the purpose of this paper only examples relevant from the biotechnological and biomedical point of view have been chosen.

CELLULAR LOCALIZATION AND MECHANISMS OF MELANIN SYNTHESIS IN PARASITIC FUNGI

The ascomycete fungus *Cryptococcus neoformans* is a parasite of the central nervous system, responsible for 5–10% lethal infections in patients with immunodeficiency syndromes (Curie & Casadevall,

1994; Mitchell & Perfect, 1995). *C. neoformans* expresses laccase which produces melanin precursors from tyrosine, DOPA, and catecholamines, abundant in the host organism (Wang *et al.*, 1995; 1996). However, the nature of the pigment produced *in vivo* under such conditions (eumelanin or other products of catecholamine oxidation) remains controversial (Liu *et al.*, 1999). *C. neoformans* laccase is localized mainly in the outer layer of the cell wall, and this is also the place of melanin deposition. Such a localization is beneficial for the fungus for two reasons (Wang *et al.*, 1995; 1996). First, it prevents the potentially toxic side-products and intermediates of melanogenesis from penetration to the cytoplasm. In higher animals this is achieved by localization of the whole process of melanogenesis in separate organelle — melanosomes, within the pigment-generating cells, melanocytes (Nordlund *et al.*, 1998). Secondly — the cellular wall deposition of melanin makes it a convenient barrier and a defence against toxins (e.g. drugs, antibiotics) and other harmful factors which melanin may neutralize. And indeed, melanotic strains of *C. neoformans* are much more resistant to fungicides and antibiotics (amphotericin B, fluconazole, caspofungin) than non-pigmented ones (Ikeda *et al.*, 2003). Caspofungin and amphotericin B are bound in the outer layer of the cell wall of the fungus, so only a limited amount is able to penetrate to the cytoplasm (Ikeda *et al.*, 2003).

Melanized cell wall of *C. neoformans* is unusually durable. It maintains its paramagnetic properties, structure, shape and size of the parasitic cell even after a drastic treatment destructive to amelanotic cells (boiling in 6 M HCl, treatment with 4 M guanidine isothiocyanate), whereafter fungal cells can still be observed as the so called “melanin ghosts” (Wang *et al.*, 1996). It raises the intriguing question about the existence and the mechanisms of fungal melanin degradation *in vivo*. Such a phenomenon seems to occur for endogenous melanins (Plonka *et al.*, 2005).

Melanin production correlates with enhanced virulence of other facultatively parasitic Ascomycetes. *Paracoccidioides brasiliensis*, *Sporothrix schenckii* and *Exophiala (Wangiella) dermatitidis* can serve as examples (Schnitzler *et al.*, 1999; Romero-Martinez *et al.*, 2000; Gomez *et al.*, 2001). These fungi reside in soil where they grow in the form of free-living mycelium. After rising the temperature to 37°C or *in vivo* in the host organism they form conidia covered by a melanized cell wall. This phase is called the “yeast” form, in contrast to the free-living mycelium, and the whole phenomenon is described as thermodimorphism. Due to the presence of pigment such fungi are often called “black yeast”.

Paracoccidioides brasiliensis causes dangerous, sometimes fatal systemic paracoccidiomycoses,

mainly in the South America (Gomez *et al.*, 2001). It contains laccase localized probably in the cell wall. In the presence of DOPA or other catechol substrates in the environment it synthesizes DOPA-melanin, however, in the absence of phenolic compounds it is able to produce DHN-melanin from tetrahydroxynaphthalene using the pentaketide pathway. In *Sporothrix schenckii* this pathway leads to melanization of conidia with the cell wall covered from the outside with melanin-like granules (Romero-Martinez *et al.*, 2000). This fungus is often found on rose prickles and causes sporothrichosis called “Rose-picker’s disease”. In Europe it is occasionally diagnosed in flower shop employees selling roses planted in tropical countries. The presence of melanin in the cell wall protects the conidia against digestion by proteases and hydrolases secreted by competitive microorganisms or against bacterio- and fungicidal proteins of animal origin, such as defensins, magainins or protegrins (Doering *et al.*, 1999; Rosas & Casadevall, 2001). The protective role of melanin is mainly based on the inactivation of these proteins on the cell surface or on the prevention of their contact with intracellular substrates.

The DHN pathway of melanin biosynthesis is very common in the Fungi kingdom, utilized, among others, by many fungal parasites of higher plants (Jacobson, 2000; Langfelder *et al.*, 2003; Thoma, 2003). It should be recalled that members of the PKS family, the key enzyme in this pathway, have been identified not only in fungi, but also in bacteria, and even in protozoa (Kroken *et al.*, 2003; Snyder *et al.*, 2003).

BIOCHEMICAL MECHANISMS OF VIRULENCE OF MELANOTIC PARASITIC FUNGI

The ability to produce melanin as an important factor of virulence is well documented and confirmed *in vivo* for *C. neoformans*. Mice infected with the parasite devoid of laccase and therefore unpigmented, survived significantly longer than the ones infected with the melanized fungus (Williamson, 1997; Barluzzi *et al.*, 2000). The virulence could be easily restored by transfection with the *CNlac1* plasmid containing the laccase gene (Williamson, 1997). Melanin was found to protect the parasite from ROS and RNS (reactive nitrogen species) produced during the oxygen burst by activated host macrophages (Wang & Casadevall, 1994b; Jacobson *et al.*, 1995; Wang *et al.*, 1995).

Due to its electrochemical properties, melanin may act both as an electron donor and electron acceptor (McGinness *et al.*, 1974; Menter & Willis, 1997). Therefore, it is not only an effective free radical “sweeper”, but also an ion-exchange resin able

to bind iron ions (Pilas *et al.*, 1988). The limited iron ion pool decreases the possibility of Fenton reaction and the formation of extremely reactive hydroxyl radical from superoxide radical anion and hydrogen peroxide (Pilas *et al.*, 1988). It has also been shown that melanin imitates action of superoxide dismutase (SOD), thus additionally limiting oxidative stress (Korytowski *et al.*, 1986). More importantly, it has recently been shown that through oxidation of DHI-CA and DHI residues to respective semiquinones, DOPA-melanin scavenges also RNS, mainly the extremely toxic radical of nitrogen dioxide ($\cdot\text{NO}_2$) produced by disproportionation of nitrite (NO_2^-) in low pH (Reszka *et al.*, 1998). This mechanism is probably responsible for the survival of melanotic *C. neoformans* strains in acidic solutions of NaNO_2 (Wang & Casadevall, 1994b).

Melanotic *Sporothrix schenckii* strains are more resistant to UV, NO and H_2O_2 than amelanotic ones (Romero-Martinez *et al.*, 2000). Non-pigmented cells are also more prone to killing by murine macrophages and human monocytes because of their high vulnerability to oxygen burst and activation of nitric oxide synthase (Romero-Martinez *et al.*, 2000).

Exophiala dermatitidis, another representative of "black yeast" is responsible for pheohyphomycoses (e.g. the ones related to diabetes) (Schnitzler *et al.*, 1999). It also preferentially infects the nervous system of immunodeficient patients and exploits the protective function of DHN-melanin as well as carotenoids — torulene and torulorodine — against strong oxidants (hypochlorite, permanganate) and free radicals. Carotenoids reveal mainly a screening activity while melanin can actively neutralize the oxidants (Schnitzler *et al.*, 1999). Melanized strains of *E. dermatitidis* are more resistant to killing by neutrophils than the amelanotic strains. Melanin protects the cells against ROS and lysis in phagosomes, although it does not affect the efficiency of phagocytosis, nor the kinetics of oxygen burst (Schnitzler *et al.*, 1999).

ACTION OF MELANIN ON THE IMMUNOLOGICAL SYSTEM OF THE HOST

The present examples illustrate an extremely intriguing aspect of the general biological implications of melanin production and activity. To get the whole picture, it should be supplemented with the fact that melanization is one of the effector mechanisms of invertebrate immunity (Nappi & Ottaviani, 2000). It can be argued whether melanization of the infected tissue is just a side-effect or the protection against the on-going inflammatory processes and the corresponding production of various active oxygen or nitrogen species, or an active defence against the

pathogen, e.g. leading to the isolation of the invader from the rest of the organism or to inactivation (adsorption) of harmful and toxic products secreted by the parasite (Marmaras *et al.*, 1996). Whatever is said, melanization is a sword of two edges, which in some cases may limit the parasite invasiveness, and in some others — the immunological mechanisms of the defence.

Melanins turned out to be immunogenic. Mice immunized with melanin extracts from the cells of *C. neoformans* produced specific anti-melanin antibodies (Nosanchuk *et al.*, 1998; Rosas *et al.*, 2001). The authors claim that melanins belong to the T-independent antigens, which can induce an immunological response by binding directly to the immunoglobulin-like receptors on the surface of B-lymphocytes (Nosanchuk *et al.*, 1998).

Various kinds of melanins turned out to reveal immunomodulatory activity through the inhibition of pro-inflammatory cytokine production by T-lymphocytes and monocytes, as well as fibroblasts and endothelial cells (Huffnagle *et al.*, 1995; Arramidis *et al.*, 1998; Barluzzi *et al.*, 2000; Mohagheghpour *et al.*, 2000).

The presence of melanin in the cell wall is correlated with less efficient phagocytosis, both in the case of *C. neoformans*, and *S. schenckii* (Nosanchuk & Casadevall, 1997; Romero-Martinez *et al.*, 2000). A likely explanation is that phagocytosis is impaired by the decrease of the negative electric charge of the cell wall, which is caused by melanin deposition (Wang *et al.*, 1995; Nosanchuk & Casadevall, 1997).

Finally, melanins of the cryptococcal cell wall activate supplement *via* the alternative way (Rosas *et al.*, 2002). Deposition of the C3 fragments on the surface of the polysaccharide envelope of *C. neoformans* is obligatory for the effective phagocytosis and removal of the fungus by phagocytes. Melanization, however, does not influence the kinetics of this deposition, nor the final amount of the deposited C3 fragments (Rosas *et al.*, 2002).

MELANIN IN THE PROTECTION AGAINST PREDATORY PROTOZOA AND NEMATODES

Development of such an intricate mechanism of virulence as the one in *C. neoformans*, an organism which is only a facultative parasite, and which does not need an animal host to successfully complete its life cycle, is puzzling. It most probably evolved as an adaptation of a free-living organism to environmental stress conditions. Melanin production offers protection from UV light and ionizing radiation, and resistance to heat or cold and activity of inorganic antimicrobial compounds, such as silver nitrate (Wang & Casadevall, 1994a; Rosas

& Casadevall, 1997; Garcia-Rivera & Casadevall, 2001; Nybakken *et al.*, 2004). Such a phenomenon was also discovered in an arctic lichen *Cetraria islandica*, where fungal melanin produced in the sun highly lowers the cortical transmittance for UV-B (Nybakken *et al.*, 2004).

One of the most probable and interesting hypotheses concerning the evolutionary transformation of melanin from such an environmental protector to a virulence factor supposes that the virulent properties appeared as a means of protection against predatory amoebae of the genus *Acanthamoeba*, for which such fungi as *C. neoformans* are a natural prey in the soil environment (Steenbergen *et al.*, 2001). Amoebae, like macrophages, phagocyte fungal cells and digest them in their phagosomes using lysosomal enzymes. The presence of a polysaccharide envelope decreases the phagocytic effectiveness, most probably due to its negative charge (Nosanchuk & Casadevall, 1997). Cells devoid of the envelope are more prone to phagocytosis, but among them, the melanized ones survive longer after incubation with amoebae (Steenbergen *et al.*, 2001).

The strains of *C. neoformans* most virulent for mice, i.e. the ones with the polysaccharide envelope, active laccase and phospholipase, are able to survive and proliferate inside phagosomes and cause death of the amoeba or macrophage which ingested the fungus (Steenbergen *et al.*, 2001). Moreover, the same mechanisms of virulence observed during cryptococcal infections in mammals, including melanin production, are also responsible for the death of the predatory nematode *Caenorhabditis elegans* feeding on *Cryptococcus* sp. fungi (Mylonakis *et al.*, 2002). Melanized strains of cryptococci are much more effective in infection and killing the nematodes in DOPA-containing media than the ones devoid of laccase (Mylonakis *et al.*, 2002).

MELANIN IN THE PENETRATION OF THE HOST CELL WALL BY PARASITIC FUNGI

Pathogenic fungal parasites of plants often develop melanized conidia or appressoria — specialized organs which facilitate adhesion to the plant surface and penetration of the hypha into the host tissues (Howard & Valent, 1996; Money, 1999). DHN is synthesized in the cytoplasm and transported to the cell wall, where it is polymerized by laccase (Thomma, 2003). The thickest melanin deposition is found in the outer layer of the wall, similarly to the situation in *C. neoformans*. *Cochliobolus* sp. and *Alternaria* sp. produce pigmented conidia and small, non-pigmented appressoria, whereas *Colletotrichum* sp. and *Magnaporthe* sp. — non-pigmented conidia and melanized appressoria (Takano *et al.*, 1997). In the

latter genus, albinotic mutants lose virulence and the ability to penetrate the plant cell wall — thus the ability to produce melanin appears an important adaptation to the parasitism (Howard & Valent, 1996). Takano *et al.*, (1997) showed that albinotic mutants of *Colletotrichum lagenarium*, devoid of the PKS gene (*Pks-*), in contrast to the wild type, were not able to infect cucumber leaves and to penetrate their cellulose wall. This ability was, however, restored by transfection of the parasite with the gene of PKS from *Alternaria alternata*, which restored appressorium pigmentation (and in the species of origin caused pigmentation of conidia). Although such “rescued” mutants were still less effective in the infection of cucumber leaves, most probably due to the lower melanin content than in the wild type, this experiment confirmed the key role of the pigment in the parasitic properties of the fungus (Takano *et al.*, 1997).

Appressorial cells reveal unusual turgor and mechanical resistance. Their osmotic pressure was estimated to reach 8 MPa. In *Magnaporthe grisea* the force which acts on the plant cell wall in the apical part of the appressorium equals 8 $\mu\text{N}/\mu\text{m}^2$, and in *Colletotrichum graminicola* — about 17 $\mu\text{N}/\mu\text{m}^2$, which is roughly equivalent to 8 tonnes on the surface of a human palm (Bechinger *et al.*, 1999). Such a high osmotic pressure is reached thanks to the extraordinary concentration of glycerol (over 3 M) in the appressorium (de Jong *et al.*, 1997; Latunde-Dada, 2001). To maintain such a high intracellular concentration, an unusual density of cell membranes and walls must be achieved. Howard *et al.* (1991) showed that melanin present in the cell wall of *M. grisea* decreases the pore diameter below 1 nm, whereas water-permeability remains unaffected. At the same time, the mechanical resistance of the cell wall profoundly increases due to melanization. Fungi from the genus *Magnaporthe* or *Colletotrichum* which attack rice, coffee plants, leguminous plants and other valuable species, are able to penetrate even intact cuticula using their melanized appressoria (Howard & Valent, 1996; Latunde-Dada, 2001). *Cochliobolus* or *Alternaria* — fungi with non-pigmented appressoria — do not achieve high enough turgor to penetrate tissue, therefore they need a specialized enzymatic apparatus degrading the plant cell wall (Bechinger *et al.*, 1999). These species, however, produce pigmented conidia, which improves their resistance to various dangerous factors of the environment (Deising *et al.*, 2000).

MYXOMYCETES

These organisms, including especially the true (acellular) slime moulds, combine features of

both protozoa and fungi (McCormick *et al.*, 1970; Lenaers *et al.*, 1988; Roger *et al.*, 1996). On the one hand, as relatives of primitive fungi, they are able to produce strongly melanized spores (McCormick *et al.*, 1970; Rakoczy & Panz, 1994; Chet & Hutterman, 1977; Loganathan & Kalyanasundaram, 1999). But on the other hand, accumulation of melanin or melanin-like pigments may also occur in the vegetative form of their life cycle, i.e. in the plasmodium, when the slime mould is similar to a multinuclear amoeba. This feature has so far been reported only in one species, *Physarum nudum*, which produces the plasmodial melanin when exposed to visible light (Plonka & Rakoczy, 1997). This seems to be a primordial reaction to light, which might have evolved to sun-tanning observed, e.g., for humans. Therefore, these microorganisms may in fact be closer to the animal melanogenetic phenomena than any other mentioned in the current review.

CONCLUSIONS

The ability to produce melanin is widespread among microorganisms. From the chemical point of view the only common feature of microorganismal melanins is being a product of oxidative polymerization of various phenolic substances, thus melanins form a quite heterogeneous group of biopolymers. As a consequence, melanogenesis can serve as an example for evolutionary convergence – the final product can be achieved on various metabolic pathways, even in one taxon. As mutations in genes determining melanin production are rarely lethal, at least under *in vitro* conditions, this feature seems not to be indispensable for life, although it creates a powerful adaptive advantage. The possible scenario of evolution might include, first, extracellular autooxidation of some phenolic compounds and amino acids due to the appearance of oxygen in the atmosphere, then, to improve the environmental conditions, active secretion of the substrates of melanogenesis and of enzymes supporting this process, followed by a gradual adaptation to the intracellular control of the process, to export or storage of the product. In parallel “secondary” biological functions of melanins might have developed.

Besides mimicry, signalling, as well as protection against UV and visible light, free radicals, extreme temperatures, and maintaining a proper balance of metal ions, there are three important functions of melanins particularly attributed to microbial organisms:

1. The function of alternative electron acceptors or carriers, making it possible to produce energy in processes analogical to oxidative phosphorylation, but under anaerobic conditions.

2. The panenvironmental function of soil melanins and humic substances, products of enzymes contained in secretomes of various soil microorganisms (e.g. streptomycetes). The most important aspect of this function is lowering the vulnerability of the soil microecosystems to UV irradiation, but also maintaining the proper ion balance in the soil. It is hardly possible to imagine a natural ecosystem which supports the existence of higher plants and is devoid of humic substances.

3. The function of a factor of virulence in pathogenic microorganisms lowering the vulnerability to the defence mechanisms of the hosts. Important for both Pro- and Eukaryotic pathogens, it draws one's attention in quest of specific targeting of individual microbial virulence factors as an alternative antimicrobial strategy (Dubin *et al.*, 2005).

In contrast to bacteria, whose melanogenic enzymes are usually secreted to the environment, in the case of eukaryotic microbial enzymes melanin production is usually connected with modification of the cellular wall. The molecular mechanisms of exploitation of melanin by parasitic fungi are refined and they must be a result of a long co-evolution. Consequently, these mechanisms are not good candidates for the primary phenomena from which the mechanisms of melanogenesis in higher Eukaryota evolved. As myxomycetes are able to both deposit melanin in the cell wall of the spores, and to produce melanin in the plasmodium after exposure to light, they seem to be closer to the primordial melanogenic Eukaryota. A closer examination of protozoa will surely reveal some new aspects of animal melanogenesis, and, perhaps, shed new light on the evolutionary origin of this phenomenon.

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