

The *mela* gene is essential for melanin biosynthesis in the marine bacterium *Shewanella colwelliana*

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The surface-adhering, Gram-negative marine bacterium *Shewanella colwelliana* synthesizes a red-brown melanin in the late stage of exponential growth in laboratory culture. Previous studies identified a single gene, *mela*, from *S. colwelliana* that could impart the ability to produce melanin to an *E. coli* host. However, these studies did not demonstrate a requirement for *mela* during melanization in *S. colwelliana*. In this paper, genetic analyses, using a broad host range conjugation system to generate specific lesions, reveal that *mela* null mutants fail to synthesize pigment. The wild-type *mela* gene provided *in trans* on a low copy number plasmid complemented these null mutations, as well as a spontaneous pigment variant, to wild-type melanin synthesis. Polyclonal antibodies, raised against a MelA–LacZ fusion protein, were used to confirm the presence of the *mela* gene product in wild-type *S. colwelliana* and verify its absence in the non-pigmented mutants. In addition, detection of the MelA protein over the course of growth in batch culture revealed a constant steady-state level of MelA protein, suggesting that the timing of melanization and the quantity of melanin synthesized is not controlled at the level of *mela* expression.

Introduction

Shewanella colwelliana is a periphytic marine bacterium isolated in association with the eastern oyster *Crassostrea virginica* (Weiner *et al.*, 1985). In laboratory culture *S. colwelliana* strain D (hereafter referred to as *S. colwelliana*) synthesizes a brownish-red melanin. Intermediates of melanization in *S. colwelliana* are thought to have a key role in the chemical signalling that stimulates free-swimming oyster larvae to colonize surfaces in the marine environment (Bonar *et al.*, 1986; Coon *et al.*, 1985; Weiner *et al.*, 1989) and it has been demonstrated that factors that affect the levels of melanin synthesis in *S. colwelliana* cultures also influence the ability of those cultures to induce oyster settlement *in vitro* (Fitt *et al.*, 1989). Melanization normally occurs towards the end of exponential growth, but this can be delayed significantly by the presence of 1–2% glucose in the culture medium. Formation of the pigment under either condition is augmented strongly by additional tyrosine.

Melanin is a broadly defined group of dark pigments identified in at least some species of virtually all

organisms. Although melanogenesis in animals has been investigated intensively (Hearing & Tsukamoto, 1991), very few bacterial melanization systems have been thoroughly studied and even fewer have been defined at the molecular level. Among this limited group, the genes for tyrosinases from several species of *Streptomyces* have been cloned, sequenced and shown to encode proteins with identifiable sequence similarity to mammalian tyrosinases (Bernan *et al.*, 1985; Huber *et al.*, 1985). Melanization in *Streptomyces* spp. appears to be a highly regulated process and may be controlled allosterically by the level of intracellular copper (Lee *et al.*, 1988; Tseng & Chen, 1991), and at the level of gene expression in response to the concentration of copper (Held & Kutzner, 1990), various amino acids (Cramer *et al.*, 1972; Katz & Betancourt, 1988), and ammonia (Held & Kutzner, 1990).

In brown-pigmented strains of *Rhizobium leguminosarum* biovar *phaseoli*, melanization is mediated by the *mela* gene product, and melanin is thought to play a role in nitrogen fixation in nodules (Borthakur *et al.*, 1987). Accordingly, *R. leguminosarum* bv. *phaseoli* *mela* is regulated in response to nitrogen levels not only by the *nifA* transcriptional regulator (Hawkins & Johnston, 1988) but also by a second gene designated *melC* (Hawkins *et al.*, 1991). Interestingly, catechol melanin

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formation in another nitrogen-fixing bacterium, *Azotobacter salinestris* (formerly *A. chroococcum*), is also controlled in response to ammonia concentration, and may be involved in oxygen scavenging during nitrogen fixation (Shivprasad & Page, 1989). The soil bacterium *Vibrio tyrosinaticus* and a hypertoxic mutant of *V. cholerae* are reported to synthesize melanin (Ivins & Holmes, 1981; Pomerantz & Murthy, 1974). Although the regulation of melanization in *Vibrio* spp. is unclear, recent studies have shown that under non-optimal culture conditions wild-type *V. cholerae* synthesizes melanin, suggesting a stress-responsive control in this bacterium (Coyne & Al-Harthi, 1992). No sequence information is currently available for any of the genes required for melanin synthesis in these bacteria.

A number of bacteria are reported to produce melanin from phenolic degradation products, such as 3,4-dihydroxyphenylacetate, that accumulate during the catabolism of tyrosine (Barnett & Hageman, 1983; Müller, 1985; Trias *et al.*, 1989). Tyrosine degradation generally requires multiple catalytic steps to convert tyrosine to melanin precursors. Some bacteria are known to accumulate homogentisic acid via a complex reaction mechanism involving the transamination of L-tyrosine to *p*-hydroxyphenyl pyruvate, followed by conversion of this compound to homogentisate (Gottschalk, 1986). Homogentisate can spontaneously oxidize to form melanin, as it does in alkaptonuric humans (Menon *et al.*, 1991). Again, no sequences for the genes involved in catabolic synthesis of melanin in bacteria have been reported.

To begin the study of the biosynthesis of melanin and its precursors, a *S. colwelliana* gene sufficient for melanin biosynthesis (*mela*) in *E. coli* was cloned, sequenced and expressed in *E. coli* (Fuqua *et al.*, 1991). The *mela* gene is expressed as a low abundance, monocistronic transcript encoding a predicted protein of 39.5 kDa. The deduced protein sequence shows no significant similarity to tyrosinases, a class of copper-binding enzymes known to mediate melanization in some animals, fungi and bacteria (Lerch, 1981). In addition, spectrophotometric tyrosinase assays with crude lysates of *S. colwelliana* or *E. coli* expressing *mela* do not detect this activity. This and other evidence suggests that *mela* directs melanization from tyrosine via an alternative mechanism. Although the *mela* gene alone mediated melanization in *E. coli*, this does not rule out the possible requirement for an additional heretofore uncharacterized enzyme(s) in *E. coli* and *S. colwelliana*, that converts tyrosine to the proper substrate for MelA. The Mel⁺ phenotype of *E. coli* harbouring *mela* demonstrates that the gene can impart the ability to synthesize melanin to a foreign host, but does not elucidate directly its role in *S. colwelliana* melanization. In the current study, genetic and molecular

analyses provide direct and indirect evidence for the integral role of the *mela* gene in *S. colwelliana* melanization. Insights into the relationship between *mela* expression and the timing of melanin synthesis in culture, as well as the influence of glucose and tyrosine are also presented.

Methods

Strains and plasmids. Strains of *E. coli*, *S. colwelliana*, and plasmids are listed in Table 1.

Media and general microbiology. *E. coli* strains were grown in Luria-Bertani (LB) media or M9 minimal medium under standard conditions. *S. colwelliana* strains were grown in Marine Broth (MB) 2216 (Difco) at 25 °C with vigorous aeration. Standard antibiotic concentrations were used for *E. coli* (Sambrook *et al.*, 1989), while for *S. colwelliana* the concentrations were; chloramphenicol (Cm), 2.5 µg ml⁻¹; rifampicin (Rif), 150 µg ml⁻¹; and tetracycline (Tc), 100 µg ml⁻¹. Melanin production was assayed on either LB or MB 2216 media supplemented with 5 mg L-tyrosine ml⁻¹.

Conjugal mating between *E. coli*/λpir derivatives and *S. colwelliana* recipient strains was performed by a patch-mating protocol. Equal swabs of donor and recipient strains were thoroughly mixed on the surface of a MB 2216 agar plate (conjugation was blocked on LB agar) to form a 1 cm patch in the centre of the plate, and mating was allowed to proceed for 24 h at 25 °C. The mating patches were washed twice in 1 ml of MB 2216, resuspended in a final volume of 1 ml, and serial dilutions were incubated on the appropriate selective media at 25 °C for 3–4 d. Negative controls with identically prepared cultures of donors or recipients alone were included.

DNA manipulations. Transformation, DNA isolation, restriction enzyme digestion and modification of DNA were performed essentially as described by Sambrook *et al.* (1989). Double-stranded DNA was sequenced with Sequenase Version 2 and [α-³⁵S]dATP (Amersham) as prescribed by United States Biochemicals. DNA probes for hybridizations were generated using the polymerase chain reaction (PCR; Gelfand *et al.*, 1990) and pairs of synthetic oligonucleotides (synthesized on a Milligen 7500 DNA synthesizer). The oligonucleotides used for PCR were 5'-TCGCCAGATGGTACTTTCTG-3' and 5'-GAAGAGTTGTGGATAGTTCA-3' to amplify a 700 bp probe for the 3' end of *mela* (GenBank accession no. M59289), Universal and Reverse Primer for amplification of the 600 bp insert in an EcoRI deletion construct of pCAT19 (Fuqua, 1992), and 5'-ATCCGCCTCCATCCAGTCT-3' and 5'-TGAAGATCAGTTGGGTGCAC-3' to generate a 535 bp β-lactamase probe from pBR325 (Bolivar, 1978).

Southern blot analyses. An alkaline, capillary blotting protocol (Reed & Mann, 1985) was used to transfer DNA separated on 0.8% agarose gels to Zetaprobe activated nylon membranes (Bio-Rad). Membranes were air-dried prior to rehydration in 6× SSPE (1× SSPE is 150 mM-NaCl, 10 mM-NaHPO₄, 10 mM-EDTA, pH 7.4) and pre-hybridization in a solution of 6× SSPE, 0.1% SDS, 0.5% skim milk and 100 µg denatured salmon sperm DNA ml⁻¹ (SSD) at 65 °C for at least 1 h. Following this incubation, the prehybridization solution was poured off and a denatured, digoxigenin-labelled DNA probe (Genius Random Primer labelling system, Boehringer-Mannheim) in 8 ml 6× SSPE, 100 µg SSD ml⁻¹ was added to the membrane, prior to incubation at 65 °C for 12 h. The membrane was subsequently washed twice in 2× SSPE, 0.01% SDS at 65 °C for 5 min followed by two washings in 0.2× SSPE, 0.01% SDS at 65 °C for 5 min. The chemiluminescent development reaction was performed as prescribed

Table 1. Strains and plasmids used and generated in this study

Strain or plasmid	Characteristics	Reference/source
<i>E. coli</i>		
AB705	<i>lacZYA</i> deletion strain	Luria <i>et al.</i> (1960)
JM101	α -Complementation strain	Messing (1979)
SY327/ λ pir	Host for pGP704-derivative cloning and maintenance, λ pir <i>recA56</i>	Miller & Mekalanos (1988)
SM10/ λ pir	Conjugal strain for pGP704 transfer, λ pir <i>recA</i> :RP4-2-Tc::Mu	
<i>Shewanella colwelliana</i>		
D	Diffusible melanin	Weiner <i>et al.</i> (1985)
DR5	Rif ^R derivative of D	This work
PIM1	Spontaneous Mel ^D mutant	
PIM1R	Rif ^R derivative of PIM1	
C72	Cm ^R Mel ^D <i>melA</i> ::CAT19 pGB3A7 insertion	
C75	Cm ^R Mel ⁻ <i>melA</i> ::CAT19 marker replacement	
Plasmid		
pUC19	Cloning vector	Yanisch-Perron <i>et al.</i> (1985)
pBR325	Cloning vector	Bolivar (1978)
pLKC480-482	Km ^R Ap ^R <i>lacZY</i> fusion cassette series	Tiedeman & Smith (1988)
pRK290	Tc ^R , broad host range IncP	Ditta <i>et al.</i> (1980)
pGP704	Ap ^R , R6K origin, mobilizable cloning vector	Miller & Mekalanos (1988)
pCAT19	Ap ^R Cm ^R , vector carrying CAT19 cassette	Fuqua (1992)
pMC3A	Ap ^R Mel ⁺ <i>melA</i> in pUC19	Fuqua <i>et al.</i> (1991)
pMC6A	Ap ^R Mel ⁺ <i>melA</i> in pUC19	Fuqua <i>et al.</i> (1991)
pMAC3	Ap ^R Km ^R Lac ⁺ <i>melA</i> ::LKC482	This work
pGB3A7	Ap ^R Cm ^R Mel ⁻ (<i>melA</i> ::CAT19)	This work
pRCAM2	Tc ^R Cm ^R	Obtained from E. J. Quintero
pRKmel	Tc ^R Cm ^R Mel ⁺ <i>melA</i> in pRK290	This work

by Boehringer-Mannheim using anti-digoxigenin Fab-alkaline phosphatase conjugate and Lumi-Phos substrate; the filters were autoradiographed. Hybridization conditions for colony blots were identical to those used for Southern blots although probes were random-primer labelled with [α -³²P]dCTP (Amersham) and washed filters were autoradiographed directly.

Generation of MelA-specific antiserum. Antiserum was raised to an affinity-purified MelA-LacZ fusion protein encoded by plasmid pMAC3 as follows. A 1 litre culture of *E. coli* AB705 harbouring pMAC3 was grown for 16 h at 37 °C. Cells were harvested by centrifugation at 16000 g (10 min, 4 °C), washed in 50 ml 1 × TE (10 mM-Tris/HCl pH 7.4, 1 mM-EDTA), centrifuged again, and resuspended in 10 ml 1 × TE. The suspension was subjected to two freeze-thaw cycles (-75 °C-25 °C) prior to sonication on ice using a Branson cell disruptor at 50% duty for a total of 15 min. Unlysed cells were removed by centrifugation at 16000 g (15 min, 4 °C), the supernatant was filtered, and the filtrate was placed on ice. Total protein was precipitated with 3 vols saturated (NH₄)₂SO₄ added dropwise with

stirring. After overnight precipitation at 4 °C, the precipitate was collected by centrifugation (16000 g, 20 min, 4 °C) and washed twice with 75% (saturation) (NH₄)₂SO₄. Following centrifugation, the pellet was dissolved in 10 ml 1 × TE and dialysed for 24 h against 1 × TE. Following dialyses, the insoluble material was removed by centrifugation (16000 g, 20 min, 4 °C), and the preparation was diluted to a concentration of 1.5 mg total protein ml⁻¹. A 1 ml Protosorb LacZ immunoaffinity column (Promega) was utilized to purify the fusion protein from 45 mg of the preparation using the manufacturer's recommended protocol and alkaline elution of the bound material in 3 ml 0.1 M-NaHCO₃/Na₂CO₃. The purity of this preparation was verified using SDS-PAGE (Laemmli, 1970) and silver staining (data not shown). Three Balb/C mice were injected intraperitoneally with 110 µg of the affinity-purified MelA-LacZ preparation resuspended in Ribi incomplete Freund's adjuvant. The mice were boosted at day 21 with another 110 µg dosage of the MelA-LacZ preparation, followed by a final immunization with 20 µg on day 28. On day 32 the mice were sacrificed and serum was collected. Western blots were immunostained with horseradish-peroxidase-conjugated goat anti-mouse secondary antibodies (Pierce) and 4-chloro-1-naphthol.

Antibodies reactive to β -galactosidase and other *E. coli* determinants were absorbed from the preparation by extensive incubation with cut squares of nitrocellulose previously treated with *E. coli* β -galactosidase (Sigma) and crude lysates of *E. coli* AB705(pUC19) in 0.1 M-sodium phosphate buffer, pH 8.0.

Results and Discussion

Melanization of *S. colwelliana* strains and *E. coli* carrying *melA*.

When *S. colwelliana* is cultured on MB 2216 agar supplemented with tyrosine, it produces the red-brown pigment melanin (Fig. 1, plate 1). Likewise, *E. coli* derivatives expressing the *S. colwelliana melA* gene (Fuqua *et al.*, 1991) synthesize the same pigment on LB agar supplemented with tyrosine (Fig. 1, plate 3), while wild-type *E. coli* does not (Fig. 1, plate 4). Significantly less pigment is synthesized on these enriched media without additional tyrosine. A defined medium for growth of *S. colwelliana* has yet to be developed, but *E. coli* strains carrying *melA* produce melanin on M9 minimal medium only when supplemented with tyrosine. The melanin produced by *S. colwelliana* and *E. coli* harbouring *melA* has been shown to conform to a number of the simple chemical criteria used to characterize melanins (Aurstad & Dahle, 1972; Fuqua *et al.*, 1991; Weiner *et al.*, 1985). Previous attempts to mutagenize *S. colwelliana* with ethylmethane sulphonate (EMS) and the acridine dye ICR 191 resulted in a number of pigment variants, all of which proved to be highly unstable (Weiner *et al.*, 1985). However, a mutant isolated from stock laboratory cultures, designated PIM1, produced an intermediate level of melanization (Mel^I) compared to *S. colwelliana*, when grown on tyrosine-supplemented MB 2216 agar (Fig. 1, plate 2). This difference was less pronounced on unsupplemented MB 2216 agar. The PIM1 mutation is highly stable and no significant

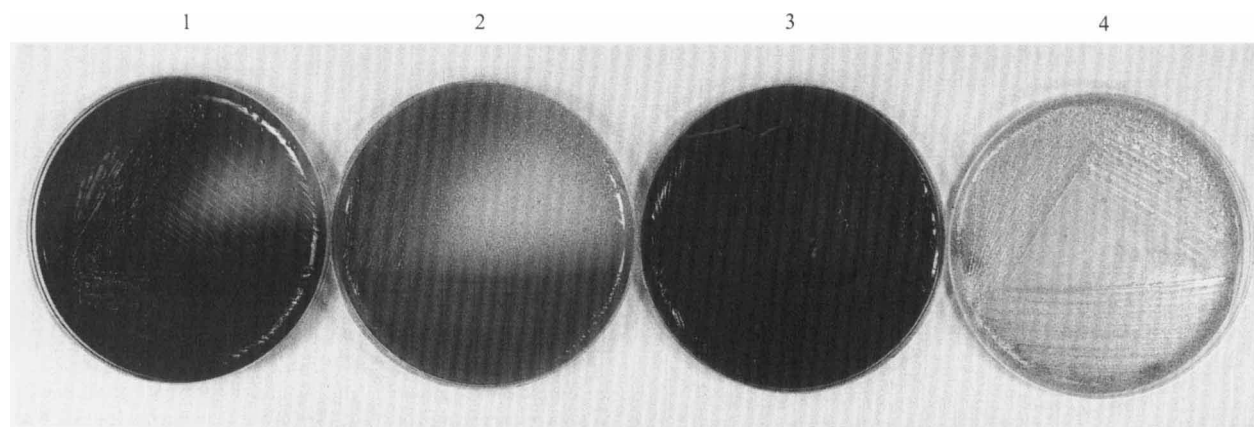


Fig. 1. Melanin synthesis on tyrosine-supplemented media. Comparison of the melanin-synthesis phenotypes of: 1, *S. colwelliana* and 2, *S. colwelliana* PIM1, grown on MB 2216 supplemented with 5 mg tyrosine ml⁻¹; and 3, *E. coli* JM101 carrying *mela*A and 4, *E. coli* JM101 carrying pUC19 on LB agar supplemented with 5 mg tyrosine ml⁻¹.

reversion to wild-type melanization has been observed in culture.

Null mutants of melaA do not synthesize melanin

To generate site-specific *melaA* mutants of *S. colwelliana*, a chloramphenicol acetyltransferase (CAT) gene cassette was inserted into the *melaA* coding sequence *in vitro*. The 1 kb CAT19 cassette (Fuqua, 1992) was cleaved with *Bam*HI and inserted in place of a 300 bp *Bgl*II fragment in the *melaA* coding region carried on plasmid pMC3A (Fuqua *et al.*, 1991), resulting in a Mel⁻ Cm^R plasmid. The vector pGP704 is a RP4-mobilizable plasmid, with an R6K origin of replication that requires the product of the *pir* gene supplied *in trans* for replication (Miller & Mekalanos, 1988). Derivatives of pGP704 will not replicate in bacteria that do not express the *pir* gene, and thus can be used to deliver mutagenic gene fragments into strains of interest for marker replacement via homologous recombination. A 2.6 kb *Bam*HI–*Nhe*I fragment, containing the CAT19-disrupted *melaA* gene and flanking DNA was inserted into plasmid pGP704 cleaved with *Bgl*II and *Xba*I, to generate the mutagenesis plasmid pGB3A7.

The CAT19-interrupted *melaA* gene on pGB3A7 was transformed into *E. coli* SM10/ λ *pir* (Miller & Mekalanos, 1988), a strain carrying a chromosomal copy of conjugal transfer (*tra*) genes required for conjugal transfer of RP4 plasmids. The resulting strain was mated with *S. colwelliana* DR5, a Rif^R mutant of *S. colwelliana*, and transconjugants were grown selectively on MB 2216 agar with Rif and Cm. Transconjugants were transferred to MB agar supplemented with Cm and L-tyrosine, and screened for synthesis of melanin. Roughly 2000 transconjugants were screened and of 350 pigment variants

obtained, approximately 10% synthesized no visible melanin, while the remaining 90% showed reduced levels of melanin synthesis (Fig. 2*a*, plates 1–3).

As the integration of pGB3A7 into the chromosomal copy of *melaA* via a single recombination event would generate a functional *melaA* gene, it was necessary to identify bona fide marker replacement mutants. However, *S. colwelliana* is resistant to β -lactam antibiotics, so that it was impossible to screen for loss of the pGP704 Ap^R resistance marker. Instead, colony hybridizations were performed with gene probes specific for β -lactamases or CAT19, and transconjugants that hybridized with the latter, but not the former, were selected for further analysis. Of the 30 transconjugants screened in this manner, four mutants hybridized as predicted for marker replacements (data not shown) and none of these synthesized visible melanin (Mel⁻). A number of mutants that hybridized with both the CAT19 and β -lactamase probe synthesized intermediate levels of melanin (Mel^I). Southern blots of genomic DNA from representatives of each class of mutants (Mel⁻ and Mel^I) were probed separately with digoxigenin-labelled probes specific for CAT19 and *melaA*. Genomic DNA from *S. colwelliana* hybridized strongly with the *melaA* probe as predicted, but did not hybridize with the CAT19 probe (Fig. 2*b,c*, lanes 1 and 2, respectively). Digests of genomic DNA from a Mel^I mutant hybridized to both the CAT19 probe and the *melaA* probe, but the number and sizes of the hybridized fragments clearly indicated a duplication of the *melaA* gene on the chromosome (Fig. 2*b,c*, lanes 3 and 4). These results suggest that the Mel^I mutants represent Campbell-type, single-crossover insertions of the pGB3A7 plasmid into the resident *melaA* gene, that may have resulted in altered expression or stability of the *melaA* locus. In contrast, a single 3 kb *Pst*I fragment and a 10 kb *Hind*III fragment from digests of genomic DNA

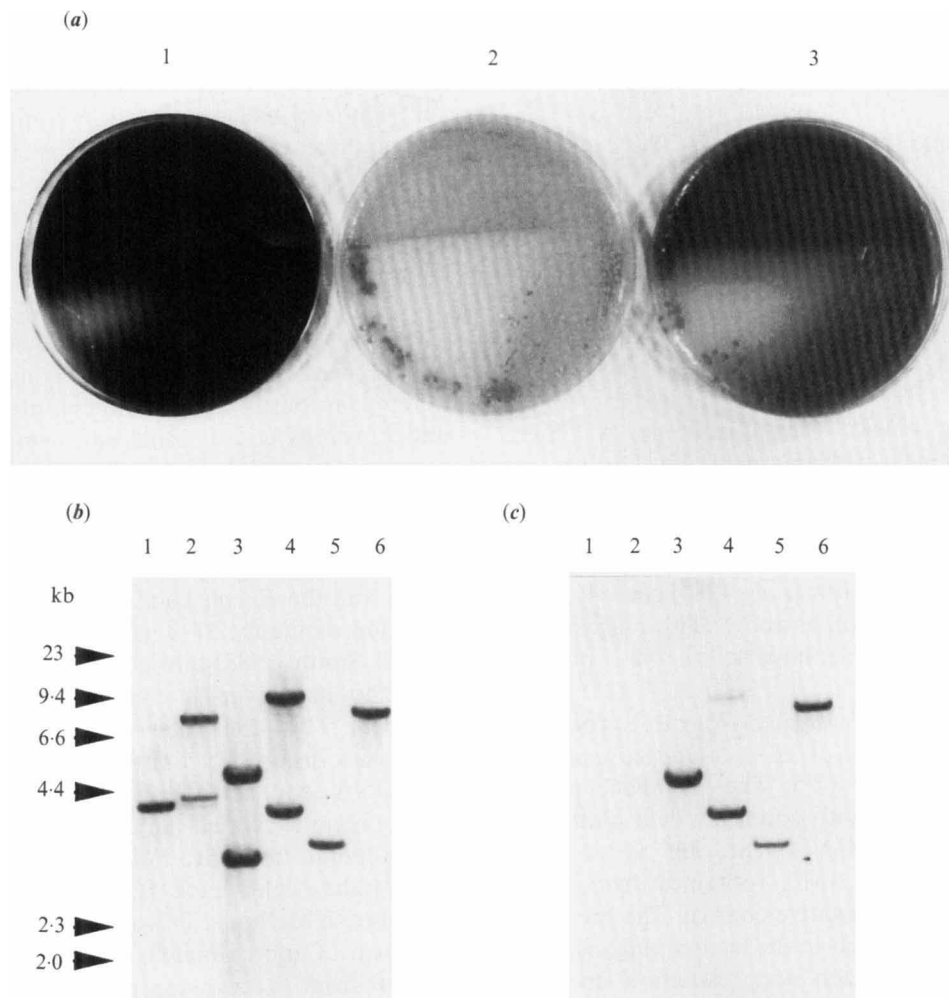


Fig. 2. Phenotypic and Southern blot analysis of *melA* mutants. (a) Melanin synthesis phenotypes of *S. colwelliana* strains 1 (Mel^+), 2, C75 (Mel^-) and 3, C72 (Mel^I) plated on MB 2216 agar supplemented with 5 mg tyrosine ml^{-1} . (b) and (c) Southern blots of identical agarose gels of genomic DNA digested with *Pst*I (odd lanes) and *Hind*III (even lanes) isolated from *S. colwelliana* (lanes 1 and 2), C71 (lanes 3 and 4) and C75 (lanes 5 and 6) probed with either *melA*-specific (b) or CAT19-specific (c) probes. DNA probes were labelled with digoxigenin using the Genius system (see Methods), and filters were exposed on X-Ray film for 2 min. The relative electrophoretic migration of *Hind*III-digested phage lambda size markers are indicated by the arrowheads.

from a $Mel^- Cm^R bla$ mutant, hybridized with the *melA* probe (Fig. 2b, lanes 5 and 6). The CAT19 probe hybridized to the identical fragments (Fig. 2c, lanes 5 and 6), indicating that the CAT19-disrupted *melA* gene had effectively replaced the resident chromosomal copy. The Mel^I and Mel^- mutants were designated C72 and C75, respectively. These results confirm that the Mel^- mutants are true marker replacements of *melA* and demonstrate the efficacy of broad host range plasmids for genetic manipulations in *S. colwelliana*. This is the first evidence that correlates the previously established ability of *melA* to direct melanization of *E. coli* with a similar function in *S. colwelliana*.

The melA gene complements melanin-deficient mutants in trans

Complementation analysis with a wild-type copy of *melA* was performed to test whether the inability of the *S. colwelliana* site-specific mutants and the spontaneous mutant PIM1 to synthesize normal levels of melanin was attributable directly to the absence of a functional *melA* gene. A broad host range plasmid derivative carrying *melA* was constructed as follows. The CAT19 cassette cleaved with *Sma*I was inserted 202 nt downstream of the *melA* coding sequence, into the single *Msc*I site in plasmid pMC6A (Fuqua *et al.*, 1991) to generate a

Table 2. Complementation analysis of *mela* mutants

Strain	Plasmid	Phenotype
<i>S. colwelliana</i> wild-type	pRCAM2	Mel ⁺
	pRK <i>mel</i>	Mel ⁺
<i>S. colwelliana</i> PIM1	pRCAM2	Mel ^l
	pRK <i>mel</i>	Mel ⁺
<i>S. colwelliana</i> C72	pRCAM2	Mel ^l
	pRK <i>mel</i>	Mel ⁺
<i>S. colwelliana</i> C75	pRCAM2	Mel ⁻
	pRK <i>mel</i>	Mel ⁺

plasmid imparting a Mel⁺ Cm^R phenotype. A 2.6 kb *Sma*I–*Hind*III fragment carrying *mela* and CAT19 was excised from this construct and the *Hind*III site was filled in using the Klenow fragment of DNA polymerase. This blunt-ended fragment was inserted into the broad host range plasmid pRK290 (Ditta *et al.*, 1980) cleaved at its single *Bgl*III site, and filled in as above. This mobilizable plasmid, designated pRK*mel*, imparted the Mel⁺ phenotype in *E. coli*.

Plasmid pRK*mel* was conjugated from *E. coli* S17-1/*λpir* into Rif^R derivatives of *S. colwelliana*, and the mutants PIM1, C72 and C75. The Tc^R Rif^R transconjugants were isolated and scored for their ability to synthesize melanin. A CAT19-carrying version of pRK290, designated pRCAM2, (obtained from E. J. Quintero) was used as a negative control. The presence of the appropriate constructs in the transconjugants was verified by plasmid isolation and restriction analysis (data not shown). All of the Tc^R Rif^R transconjugants, including the spontaneous PIM1 and the engineered Mel⁻ and Mel^l mutants, were complemented to the wild-type Mel⁺ phenotype by pRK*mel*, while the control plasmid pRCAM2 had no effect on the mutant phenotypes (Table 2). The possibility that the altered Mel⁻ phenotype in *S. colwelliana* C72 and C75 was due to polar effects of the inserted CAT19 cassette was excluded as the pRK*mel* plasmid carries only the *mela* gene. Thus, the requirement for a functional *mela* gene during *S. colwelliana* melanization was verified genetically. Complementation with *mela* is particularly informative for the PIM1 mutant, the only stable, spontaneous pigmentation variant isolated to date. The genetic analysis and examination of the abundance of the *mela* gene product in PIM1 (see below) suggest that, as in the site-directed *mela* mutants, the PIM1 defect in melanization is directly attributable to a partial or complete loss of *mela* function. While we cannot yet exclude the possibility that other gene products are also required, these genetic analyses demonstrate the essential nature of *mela* in the process of *S. colwelliana* melanization.

Detection of the *mela* gene product in *S. colwelliana* cultures

Although the *mela* gene had been shown to be essential for high-level melanization in *S. colwelliana*, there was still no direct evidence of its expression or the activity of the MelA protein in pigmenting cultures. In fact, Northern blots with oligonucleotide probes specific for *mela* failed to reveal detectable levels of the *mela* transcript in *S. colwelliana* cultures at any stage of growth (Fuqua *et al.*, 1991). Moreover, classical enzyme assays for measuring tyrosinase activity were ineffective in *S. colwelliana* and *E. coli* carrying *mela*. Repeated attempts to purify MelA from cultures of *S. colwelliana* and *E. coli* by conventional biochemical methodologies were unsuccessful. In order to circumvent these apparent limitations of detection and obtain a method for monitoring MelA protein levels, polyclonal antibodies were raised against MelA as follows. A protein fusion of MelA and the *E. coli* LacZ protein was constructed by insertion of the *lacZY* Km^R cassette pLKC482 (Tiedeman & Smith, 1988) into a *Sca*I site (nt 992, GenBank M59289) in the *mela* coding sequence to construct pMAC3. This construct was Lac⁺ Mel⁻ Km^R and encoded a fusion protein of 140 kDa (data not shown). The DNA sequence of the junction of *mela* and *lacZ* coding regions verified the predicted reading frame and fusion point in *mela*. Polyclonal antiserum directed against the MelA–LacZ fusion protein was raised as described in Methods. This antiserum specifically stained a protein of approximately 41 kDa in Western blots of extracts from *E. coli* overexpressing *mela* from the *lacZ* promoter (Fuqua *et al.*, 1991), and from *S. colwelliana* (Fig. 3a, lanes 2 and 3), but not extracts of an *E. coli* vector control (Fig. 3a, lane 1). Although not highly abundant, this was the first demonstration of the presence of the *mela* gene product in *S. colwelliana*. A larger, heavily staining band was also visible in the *S. colwelliana* extract, but does not correspond to the MelA protein and is present in immunostainings with pre-immune serum (data not shown).

The antibody preparation was used to compare extracts of *S. colwelliana*, the *mela* marker replacement mutant C75, and the spontaneous melanin variant PIM1. Equal amounts of total protein were separated by SDS-PAGE and transferred to nitrocellulose, prior to immunostaining with the anti-MelA serum. While the 41 kDa protein was stained specifically in lysates of *E. coli* expressing *mela* and *S. colwelliana* (Fig. 3b, lanes 1 and 2), the C75 and PIM1 extracts contained no detectable *mela* (Fig. 3b, lanes 3 and 4). Thus, the identity of the 41 kDa protein as the *mela* gene product was again confirmed. These results also demonstrate that the melanization-deficient phenotypes of both

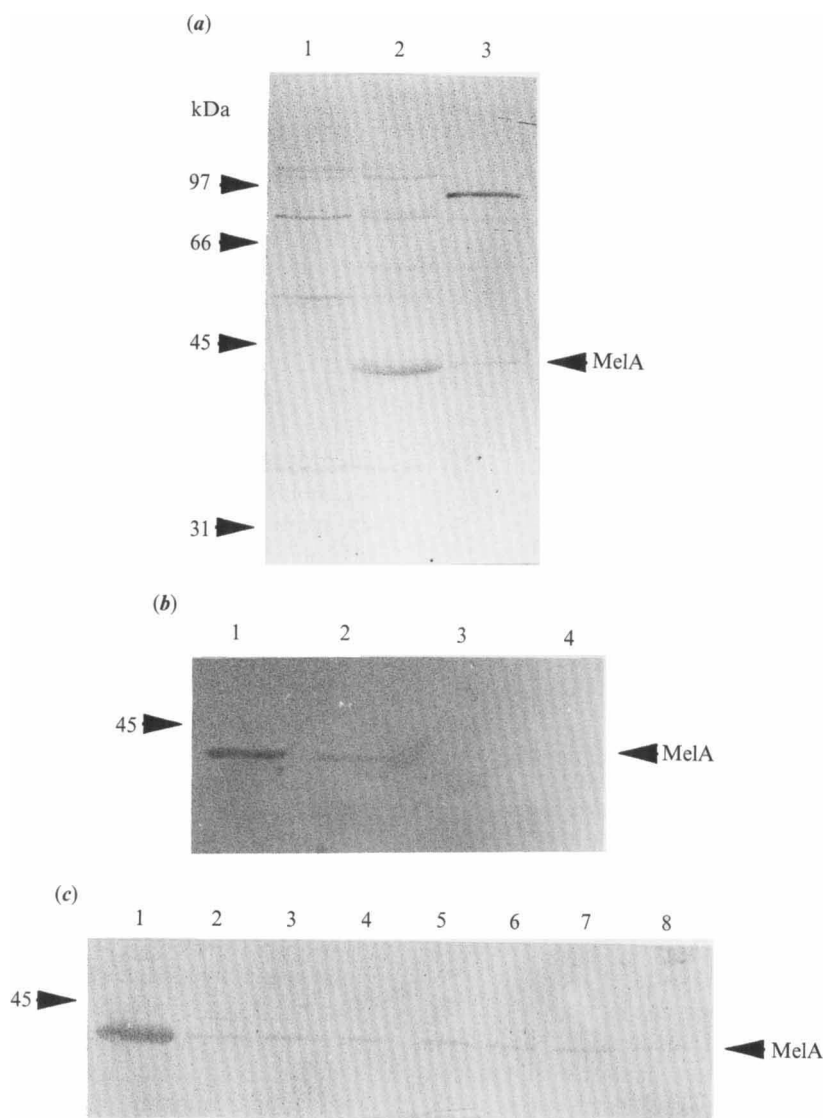


Fig. 3. Detection of the MelA protein in wild-type and mutant *S. colwelliana* lysates using polyclonal antiserum against MelA. (a) Immunostaining of electrotransfers from 12% SDS-PAGE gels loaded with whole cell lysates of *E. coli* JM101 pUC19 (lane 1), *E. coli* JM101 pMC3B Mel⁺ (lane 2) and *S. colwelliana* (lane 3). (b) Similar immunostaining of lysates from *E. coli* JM101 pMC3B (lane 1), and *S. colwelliana* wild-type (lane 2), PIM1 (lane 3) and C75 (lane 4). (c) Immunostaining of lysates of *E. coli* JM101 pMC3B (lane 1) and *S. colwelliana* from aliquots of a rapidly growing culture 11 h (lane 2), 16 h (lane 3), 20 h (lane 4), 26 h (lane 5), 34.5 h (lane 6), 42 h (lane 7) and 57 h (lane 8) after inoculation. The relative electrophoretic migration of protein size standards are indicated by the arrowheads on the left and the MelA protein is indicated on the right.

S. colwelliana PIM1 and the marker-replacement mutant C75 are due to the absence or reduced levels of MelA. Although the underlying defect resulting in the PIM1 Mel⁻ phenotype is not known, it is clearly manifested in lowered abundance of the *melA* gene product.

Additionally, recent results with a newly developed enzyme assay utilizing reverse phase HPLC and an electrochemical detection system to analyse the reaction products produced from tyrosine in *S. colwelliana* crude cell extracts have revealed an observable deficiency for

the *S. colwelliana melA* mutants when compared to the wild-type (S. L. Coon and others, unpublished).

The melA gene product and melanin precursors are present throughout batch culture growth

Similar to many other melanizing bacteria, *S. colwelliana* cultures begin producing visible levels of melanin in the late stage of growth (Fuqua, 1991; Weiner *et al.*, 1985). Thus, it was hypothesized that the timing of

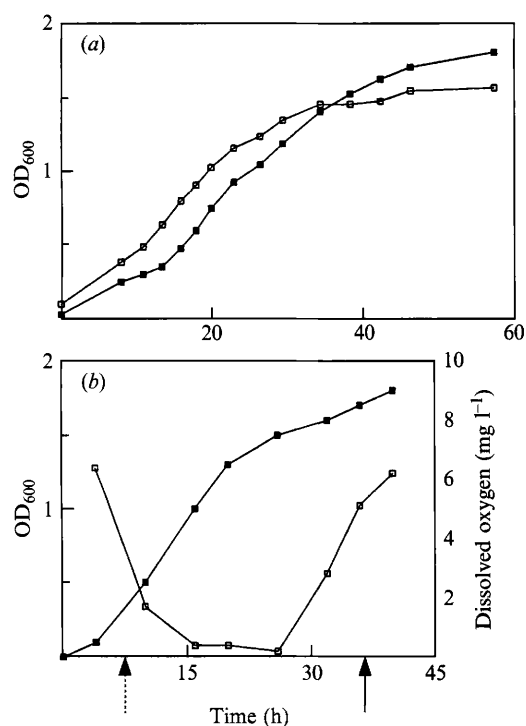


Fig. 4. Growth rate and oxygen depletion in a rapidly growing culture of *S. colwelliana*. (a) Cultures were grown in MB 2216 (□) and MB 2216 supplemented with 0.5% glucose (■), and the optical density of 600 nm was monitored. (b) Dissolved oxygen levels (□) were monitored in culture using an oxygen electrode and plotted against the time (■). The arrows indicate when melanin precursors are first detected in supernatants (†) and when visible pigmentation occurs (↑).

melanization was growth-rate regulated, possibly at the level of *mela* gene expression. Moreover, the effects noted upon addition of either tyrosine (enhanced melanization) or glucose (delayed melanization), were also postulated to be due to substrate induction and catabolite repression of *mela* expression, respectively. In the light of this observation, we wished to determine the steady-state levels of the MelA protein in batch culture. The anti-MelA serum was used to gauge the relative abundance of the MelA protein in samples from time-course batch-culture growth studies of *S. colwelliana*, standardized to equal total protein concentration. The antiserum was sensitive to 2-fold changes in MelA concentration over a range of three orders of magnitude. Surprisingly, immunostaining revealed an equal steady-state level of MelA (Fig. 3c, lanes 2–8) at all of the sample times (Fig. 4a). In addition, the presence of 5 mM-tyrosine or 1% glucose had no apparent effect on the steady-state levels of MelA in lysates (data not shown). The constant level of the *mela* gene product indicates that the late exponential stage onset of melanization observed for *S. colwelliana* cultures does not reflect an increase in the synthesis of MelA and

suggests that the *mela* gene may be expressed constitutively under the conditions tested.

To determine when melanin precursors begin to accumulate in *S. colwelliana* cultures, samples from rapidly growing liquid cultures were removed and treated with NaOH to drive polymerization of melanin precursors. In congruence with the results obtained by monitoring MelA protein levels, melanin precursors were evident in the early phase of growth (Fig. 4b, †), even though visible pigment did not form in the culture until much later (Fig. 4b, ↑). Melanin was not formed in MB 2216 controls or in cultures of the Mel⁻ mutant C75.

Polymerization of melanin is predominantly an oxidative process (Bell & Wheeler, 1986) and a reducing environment can effectively prevent the polymerization of melanin from its unstable precursors. Interestingly, in exponentially growing *S. colwelliana* cultures the dissolved oxygen concentration was below the level of detection (presumably due to rapid utilization by respiring cells), and a rise in oxygen levels as growth slowed was coincident with the timing of pigmentation (Fig. 4b). Furthermore, *S. colwelliana* cultures grown in continuous culture with saturating oxygen levels synthesize melanin constitutively (A. Enriquez & R. M. Weiner, unpublished results). However, the pH in *S. colwelliana* cultures also increases with longer incubation time (Weiner *et al.*, 1985), and this may also affect the timing of pigment formation. Collectively, these results suggest that melanin precursors are present throughout the course of growth in batch culture, albeit maintained in a non-active, perhaps reduced state. Therefore, both the *mela* gene product and melanin precursors are present throughout growth.

Interestingly, the glucose-supplemented culture remained in exponential phase longer and reached a higher final cell density than the non-supplemented culture (Fig. 4a). This observation may explain the delay in melanization usually observed in the presence of glucose, and again is probably a physico-chemical phenomenon.

Similar effects due to culture conditions have been proposed to occur in *Bacillus subtilis*, where pigmentation occurs coincidentally with sporulation (Barnett & Hageman, 1983) and is believed to be due to a shift to alkaline pH in the culture during sporulation. In *S. colwelliana*, the observed timing of melanization and the effects of tyrosine and glucose are probably attributable to dynamic changes in culture parameters during growth, including changes in redox potential or pH, over the course of growth. Alternatively, the timing of melanization and the effects of tyrosine and glucose on melanization in *S. colwelliana* could reflect the regulation of an undefined component of the melanin biosynthesis pathway that is required to drive the process to completion. For the tyrosinases, a second protein, *melC*,

is required for activity in *Streptomyces* and when the genes are expressed in *E. coli* (della-Cioppa *et al.*, 1990). However, if such an additional protein(s) is required for *S. colwelliana* melanization, it must also be present in *E. coli* (Fuqua *et al.*, 1991).

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