In vivo studies disprove an obligatory role of azurin in denitrification in Pseudomonas aeruginosa and show that azu expression is under control of RpoS and ANR

Erik Vijgenboom, Julie E. Busch and Gerard W. Canters

The role of the blue copper protein azurin and cytochrome c_551 as the possible electron donors to nitrite reductase in the dissimilatory nitrate reduction pathway in Pseudomonas aeruginosa have been investigated. It was shown by an in vivo approach with mutant strains of *P. aeruginosa* deficient in one or both of these electron-transfer proteins that cytochrome c_551, but not azurin, is functional in this pathway. Expression studies demonstrated the presence of azurin in both aerobic and anaerobic cultures. A sharp increase in azurin expression was observed when cultures were shifted from exponential to stationary phase. The stationary-phase sigma factor, σ^S, was shown to be responsible for this induction. In addition, one of the two promoters transcribing the azu gene was regulated by the anaerobic transcriptional regulator ANR. An azurin-deficient mutant was more sensitive to hydrogen peroxide and paraquat than the wild-type *P. aeruginosa*. These results suggest a physiological role of azurin in stress situations like those encountered in the transition to the stationary phase.

Keywords: azurin, redox stress, ANR, rpoS, anaerobiosis

INTRODUCTION

The blue copper protein azurin, a periplasmic redox protein and member of the cupredoxin family (Canters, 1987; Wood, 1978), has been identified in several bacteria such as *Alcaligenes xylosoxidans* NCTC 8582 (formerly *Alcaligenes denitrificans*; Hoitink et al., 1990), *Methyllobacillus flagellatum* KT (Gak et al., 1995), *Methylomonas J* (Ambler & Tobari, 1989) and *Alcaligenes xylosoxidans* NCIMB 11015 (Dodd et al., 1995). The first report of azurin was made by Horio (1958), who isolated the protein from *Pseudomonas aeruginosa* and designated it ‘Pseudomonas blue protein’. Subsequent investigations demonstrated that significant amounts of this protein could be isolated from *P. aeruginosa* grown under oxygen limitation (Yamanaka et al., 1961). The finding of azurin expression during anaerobic respiratory growth with nitrate as the final electron acceptor and in vitro experiments carried out over the years has led to the hypothesis that azurin is involved in dissimilatory nitrate reduction (Barbet et al., 1976; Silvestrini et al., 1982; Zannoni, 1989).

Cloning of the azurin gene, *azu*, from *P. aeruginosa* (Canters, 1987; Arvidsson et al., 1989) provided the possibility of investigating the mechanism underlying expression regulation and the correlation to specific growth conditions. Mapping of the transcription start sites identified two promoters, of which the proximal promoter (P1) appeared to be the stronger (Arvidsson et al., 1989). Based on sequence homology two sequence elements which could be of importance in the regulation of transcription initiation were identified in the promoter region. The P2 promoter region has similarity with the σ^N consensus sequence (Arvidsson et al., 1989; Hoitink et al., 1990), although the spacing between the strictly conserved GG and GC motifs (Morett & Buck, 1989) is not correct. RNA polymerases containing σ^N transcribe various genes involved in nitrogen metabolism, pilus production and many other functions (for a review see Merrick, 1993). A sequence with strong homology with the consensus FNR-binding site (TTGAT-N_4-ATCAA) is present upstream of P1 (Hoitink et al., 1990). The structural and functional analogue of FNR in *P. aeruginosa* is ANR, a transcription regulator which can activate transcription during oxygen limitation (Sawers, 1991; Zimmermann et al., 1991). The denitrification pathway and anaerobic arginine degra-
Table 1. List of strains and plasmids

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
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</tr>
<tr>
<td>PAO1</td>
<td>Wild-type</td>
<td>Holloway et al. (1979)</td>
</tr>
<tr>
<td>PAO6261</td>
<td>anr, 0.33 kb deletion</td>
<td>Ye et al. (1995)</td>
</tr>
<tr>
<td>S1239</td>
<td>ilvB112 leu-1 met-9 nirD(= anr), SteR</td>
<td>Van Hartingsveldt et al. (1971)</td>
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<td>RMS36</td>
<td>dnr::TcR</td>
<td>Arai et al. (1995)</td>
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<td>PSA1</td>
<td>PAO1 derivative, azu::SpcR^ SmR</td>
<td>This study</td>
</tr>
<tr>
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<td>PAO1 derivative, nirM::KmR</td>
<td>This study</td>
</tr>
<tr>
<td>PSA16</td>
<td>PAO1 derivative, azu::SpcR^ SmR nirM::KmR</td>
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</tr>
<tr>
<td>PSA30</td>
<td>PAO1 derivative, rpoS::TcR</td>
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<td>recA pro bsdR bsdM:: R4 derivative</td>
<td>Simon et al. (1983)</td>
</tr>
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<td>JM101</td>
<td>F' traD36 lac^ Δ(lacZ)M15 proA^ B'/supE thi Δ(lac–proAB)</td>
<td>Yanisch-Perron et al. (1985)</td>
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<td>JM109</td>
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<td><strong>Plasmids</strong></td>
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<td>pUCP24</td>
<td>E. coli–<em>P. aeruginosa</em> shuttle vector</td>
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<tr>
<td>pQF130</td>
<td>Promoter-probe vector with promoterless lacZ and phoA gene</td>
<td>Ronald et al. (1990)</td>
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<tr>
<td>pGP173</td>
<td>pBR322 derivative with mob site of RP4</td>
<td>Goosen et al. (1987)</td>
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<td>pEMBL18NR</td>
<td>3.5 kb EcoRI fragment harbouring nirS and nirM in pEMBL18</td>
<td>Silvestrini et al. (1989)</td>
</tr>
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<td>pDB18R</td>
<td>1.8 kb Km^ resistant fragment harbouring rpoS cloned in pTZ18R</td>
<td>Tanaka &amp; Takahashi (1994)</td>
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<td>pGC4</td>
<td>pUC8 containing the 1.3 kb PstI azu fragment</td>
<td>Van de Kemp et al. (1990a)</td>
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<td>pGC4 derivative with XbaI site (silent mutation) at position 751 bp</td>
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<td>pGP173 with PstI fragment azu::Sp^ SmR of pGC13</td>
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<td>pQF130 containing azu P1 promoter (see Fig. 1a)</td>
<td>This study</td>
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<td>pQF130 containing azu P2 promoter (see Fig. 1a)</td>
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<td>pEMBL18NR, nirM::KmR</td>
<td>This study</td>
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<td>pGC121</td>
<td>pGP173 with EcoRI fragment nirM::KmR of pGC120</td>
<td>This study</td>
</tr>
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<td>pGC135</td>
<td>pUCP24 with PstI fragment azu of pGC13</td>
<td>This study</td>
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<td>pGC139</td>
<td>See Fig. 1b</td>
<td>This study</td>
</tr>
<tr>
<td>pGC164</td>
<td>pDB18R, rpoS::TcR (1.3 kb TcR gene fragment of pBR322 in rpoS)</td>
<td>This study</td>
</tr>
<tr>
<td>pGC166</td>
<td>pGP173 with EcoRI–HindIII fragment rpoS::TcR of pGC164</td>
<td>This study</td>
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dation are dependent upon ANR for their anaerobic activation in *P. aeruginosa* (Galimand et al., 1991; Ye et al., 1995).

The electron-transport capability of azurin has been well established by in vitro experiments. The purported redox partners of azurin, cytochrome c_553 (cyt-c_553) and nitrite reductase, have been identified from in vitro electron-transfer studies, but their relevance as physiological partners has not been established in vivo (Horio, 1958; Wilson et al., 1975; Corin et al., 1983; Van de Kamp et al., 1990b; Silvestrini et al., 1994). More recently azurin has also been suggested as the primary electron acceptor for ethanol dehydrogenase in *P. aeruginosa* (Duine, 1995), for aromatic amine dehydrogenase in *Alcaligenes faecalis* (Edwards et al., 1995; Hyun & Davidson, 1995), for methylamine dehydrogenase in *Methylobacillus flagellatum* KT (Gak et al., 1995), for amicyanin in an obligate methylo troph, organism 4025 (Auton & Anthony, 1989), and as the electron donor to the copper-containing NIR of *Pseudomonas aureofaciens* (Zumft et al., 1987). Most of these suggestions have arisen from in vitro electron-transfer experiments. Only in the case of *Methylobacillus flagellatum* has it been shown that an azurin-deficient mutant, although still viable, grew much slower on methylamine than the wild-type strain (Gak et al., 1995).

In this study a detailed investigation of the expression of *azu* in several genetic backgrounds under aerobic and anaerobic conditions is presented. These data and the investigation of strains lacking azurin and/or cyt-c_553 exclude an essential physiological role of azurin in dissimilatory nitrate reduction in *P. aeruginosa*. The sensitivity of the *azu* mutant of *P. aeruginosa* towards reactive oxygen species such as hydrogen peroxide and superoxide radicals generated by paraquat point to a function for azurin in redox stress response.
Expression and role of azurin in *P. aeruginosa*

**METHODS**

**Strains and media.** Strains are listed in Table 1. LB (Luria-Bertani) medium (Miller, 1992) and defined minimal medium (MM), VB medium (Vogel & Bonner, 1956) supplemented with trace-element solution, or phosphate-buffered minimal medium were used for both *Escherichia coli* and *P. aeruginosa* cultures. Aerobic growth conditions in liquid cultures were maintained by vigorous shaking of Erlenmeyer flasks filled to a maximum of 10% of the volume and incubated at 37 °C. Anaerobic cultures were obtained in tightly capped bottles which were filled to 95% with medium. Anaerobic incubation of agar plates was done at 37 °C in an Oxoid anaerobic jar containing Oxoid AnaeroGen. Media for anaerobic growth contained 50 mM KNO₃ or 10 mM NaNO₃. MM contained 0.2% yeast extract when used under oxygen-limiting conditions. Plasmids were maintained by adding the appropriate antibiotic at the following concentrations: for *E. coli*, 100 µg ampicillin ml⁻¹, 25 µg streptomycin ml⁻¹, 15 µg gentamycin ml⁻¹; and for *P. aeruginosa*, 250 µg carbenicillin ml⁻¹, 200 µg streptomycin ml⁻¹, 200 µg neomycin ml⁻¹, 1000 µg spectinomycin ml⁻¹, 150 µg tetracycline ml⁻¹, 200 µg gentamycin ml⁻¹.

**Plasmid constructions.** The plasmids constructed and used in this study are listed in Table 1. To construct the promoter-probe plasmids, the complete *azu* promoter region was isolated as a HindIII fragment (Fig. 1a) and cloned in the *Smal* site of pQF130, resulting in pGC110. The plasmid containing the correct orientation of the fragment with respect to the lacZ gene of the vector was identified by restriction analysis. The P1 and P2 promoters could be separated by digestion with BstYI. The 236 bp BstYI–HindIII fragment contained P1 and was cloned in BglII/Smal-digested pQF130, resulting in pGC115. The 370 bp *SalI*–BstYI fragment contained P2 and was cloned in *SalI*/BamHI-digested pQF130, resulting in pGC116. The azurin gene on pGC13 was disrupted by inserting the *aadA* cassette of pUC4K (Pharmacia) in the *ApaI* site in the coding sequence of *nirM*. The complete *nirF–nirM* cluster was cloned in pGP173 as an EcoRI fragment, resulting in pGC117. For the disruption of *rpoS*, pGC164 was constructed by inserting the 1.3 kb EcoRI–Styl TeR fragment of pBR322AH (HindIII site removed by filling in the sticky ends) into HindIII/BamHI-digested pDB18R. Non-compatible restriction sites were blunt-ended. The mobilizable plasmid pGC166 was constructed by insertion of the EcoRI–HindIII fragment harbouring *rpoS::TcR* in pGP173. The *cya* expression plasmid pGC139 was constructed as outlined in the legend to Fig. 1 (b).

**Strain constructions.** Inactivation of the genomic copies of *azu*, *nirM* and *rpoS* was carried out by introducing into *P. aeruginosa* a suicide plasmid harbouring the inactivated gene. Recombination events between homologous sequences on the genome and those on the plasmid resulted in a gene replacement, leaving the inactive gene copy in the genome. All gene inactivation procedures were checked by antibiotic resistance and by Southern hybridization of total DNA with three probes: (1) the gene of interest, (2) the resistance gene and (3) vector sequences. Plasmids were introduced into *P. aeruginosa* by mating with *E. coli* strain S17-1 carrying the plasmid of interest. Strains were grown until mid-exponential phase in LB medium with the appropriate antibiotic(s), mixed in ratios varying from 1:1 to 1:5 and grown for 16 h on LB agar plates. Cells were harvested from the plates and selection for the recombinant *P. aeruginosa* was carried out by plating dilutions on MM plates with glucose and the appropriate antibiotic(s). Individual colonies were screened for their antibiotic resistance, grown in LB medium and chromosomal DNA was analysed as described above.

**Recombinant DNA technology.** Cloning of DNA fragments was carried out according to standard procedures (Sambrook *et al.*, 1989). *E. coli* strains were transformed by the method of Chung *et al.* (1989). pQF130 and pUCP24 derivatives were introduced into *P. aeruginosa* by electroporation. Electrocompetent *P. aeruginosa* cells were prepared from an over-
night culture in LB with 10 mM MgSO$_4$ which was diluted 100-fold in fresh medium and grown at 37 °C with good aeration for another 5 h. Cells were collected by centrifugation and washed twice with 13 % (v/v) glycerol, 1 mM MOPS (pH not adjusted) and the cell pellet was finally suspended in 1 ml of the same buffer. Electroporation was carried out with settings of 1250 V, 50 μF and 200 Ω. Immediately thereafter 1 ml LB with 10 mM MgSO$_4$ was added and the cells were allowed to recover for 1 min at room temperature. The antibiotic resistance was expressed at 37 °C for 60 min in a total volume of 2.5 ml LB with 10 mM MgSO$_4$ before plating on LB plates with the appropriate antibiotic. Southern hybridization was done according to standard procedures with capillary transfer of DNA to Hybond-N$^+$ (Amersham) or positively charged nylon membrane (Boehringer Mannheim) in 10 × SSC. Hybridization conditions were stringent (0.1 × SSC) and probes were labelled non-radioactively using the DIG system (Boehringer Mannheim).

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**Fig. 2.** (a) Southern blot of PstI-digested total DNA of PSA1, a strain in which the azu gene is disrupted by the insertion of the aadA gene (lane 1) and of the wild-type strain PAO1 (lane 2). The blot was probed with a randomly labelled (DIG-dUTP) 1300 bp PstI fragment containing the entire azu gene. On the left-hand side of the blot the expected genomic PstI fragments are indicated. (b) Western blot with antibodies directed against *P. aeruginosa* azurin. Cells of overnight cultures were pelleted, suspended in SDS loading buffer and samples were run on a 15% SDS-polyacrylamide gel. Lane 1, PAO1 (wild-type); 2, PSA1 (azu); 3, PSA15 (nirM); 4, PSA16 (azu, nirM). (c) Southern blot of EcoRI-digested total DNA of PAO1 (lane 1) and PSA15 (lane 2) probed with the 1500 bp SacI fragment of pEMBL18NR containing the entire nirM (cyt-c$_5$) gene. The genomic EcoRI fragment containing nirM is 3500 bp in PAO1 and 4800 bp in PSA15 as a result of gene replacement with nirM::Neo$^R$. (d) Southern blot of Smal-digested genomic DNA of PSA30 (lane 1) and PAO1 (lane 2). The Smal fragment carrying the entire rpoS gene is 1144 bp in the wild-type strain. In strain PSA30 the Smal fragment of rpoS migrates more slowly as a result of the gene replacement with rpoS::Tc$^R$. 

2856
**Expression and role of azurin in P. aeruginosa**

Enzyme assay. β-Galactosidase activity was determined directly in chloroform/SDS-treated cells as described by Miller (1992). Measurements were done in duplicate and cells from three independent cultures were used.

Western blotting. Samples were run on a 15% SDS-polyacrylamide gel and subsequently blotted to Hybond-C Super (Amersham) in the Bio-Rad Mini-Protean II system. The blot buffer consisted of 12.5 mM Tris, 96 mM glycine pH 8.3 and 20% methanol. Following the blotting procedure membranes were washed briefly in PBS (140 mM NaCl, 30 mM KCl, 8 mM NaH2PO4, 2 mM KH2PO4, pH 7.4) and incubated overnight at 4°C in PBS containing 5% milk (solution prepared from low-fat milk powder) and azurin serum (1:2500) or nitrite reductase serum (1:1000). The membranes were then washed three times (15 min per wash) with PBS and incubated for 60 min at room temperature with TBST (1 mM Tris/HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.4) containing anti-rabbit IgG alkaline phosphatase conjugate (1:5000). Staining was carried out in AP buffer (100 mM Tris/HCl, 100 mM NaCl, 5 mM MgCl2, pH 9.5) with NBT and BCIP (Gibco BRL).

**RESULTS**

Physiological role of azurin

To construct a P. aeruginosa strain deficient in azurin production we first inactivated the azu gene on plasmid pGC13 by insertion of the spectinomycin/streptomycin-resistance gene (aadA) in the middle of the coding sequence. The resulting plasmid, pGC103, was used to construct strain PSA1 by gene replacement (Fig. 2a). Western blot analysis using antibodies raised against purified azurin demonstrated that azurin was no longer produced in the mutant strain (Fig. 2b).

The growth characteristics (doubling time, maximum OD600) of PSA1 (azu) grown under aerobic conditions in LB medium were indistinguishable from those of the wild-type strain PAO1. Only upon addition of nitrite (10 mM) was a slightly reduced growth rate observed. Also under anaerobic conditions either in the presence of nitrate or nitrite the strain grew as well as did the wild-type (Table 2). An essential function of azurin in denitrification is therefore excluded. The current model for the electron-transport pathway used by P. aeruginosa during anaerobic growth with nitrate or nitrite as the final electron acceptor has both azurin and cyt-c551 as electron donors for NIR. To identify the in vivo donor we constructed strains derived from PAO1 that lack either cyt-c551 (PSA15) or both azurin and cyt-c551 (PSA16). The cyt-c551 gene (nirM) was inactivated by gene replacement with a nirM copy disrupted by the neomycin-resistance gene (Fig. 1b). In strains PSA15 and PSA16 the growth rate under anaerobic conditions was reduced by 50% in medium containing nitrate. Under the same conditions, with nitrite as the final electron acceptor, the generation time increased at least fourfold to 500 min or more and the maximum OD600 was reduced (Table 2). Disruption of the nirM gene (cyt-c551) might have a negative influence on the expression of the upstream nirS (nitrite reductase) gene. However, in both strains the expression of NIR as judged by Western blotting with antibodies directed against NIR was comparable to that in PAO1, as was the case for the incorporation of haem in NIR (data not shown). In accordance with this observation, the anaerobic growth on nitrate or nitrite of both PSA15 and PSA16 could be restored to almost wild-type performance by complementation of the genomic cyt-c551 mutation with pGC139, a plasmid harbouring and expressing nirM but not nirS. Therefore, strains PSA15 and PSA16 do produce an active nitrite reductase. All of this demonstrates that cyt-c551 is a physiological electron donor of NIR in P. aeruginosa.

Two other options for the physiological function of azurin could be excluded by analysing the growth of PSA1 and PAO1 on media with ethanol or tyramine as the sole carbon source. Both carbon sources supported growth of the wild-type strain PAO1 and the azu mutant PSA1 to a comparable level. It is, therefore, highly unlikely that azurin is the obligatory electron acceptor for ethanol dehydrogenase or aromatic amine dehydrogenase in PAO1.

**Control of azurin expression**

Since azurin is not involved in the physiological processes described above, we turned to expression studies of azu in P. aeruginosa to obtain a clue as to its physiological role. The temporal expression of azu,
Fig. 3. (a) Western blot of whole-cell extracts of PAO1 (A), PAO6261 (B) and S1239 (C). Strains were grown under aerobic conditions (10 ml LB medium in a 100 ml Erlenmeyer flask with a cotton plug and incubated in a New Brunswick model G25 incubator shaker at 250 r.p.m.) in LB medium at 37 °C. Samples were taken at an OD_{600} (as determined by a Shimadzu UV-2101 PC UV-VIS scanning spectrophotometer) of 0.4 (ML, mid-exponential-phase samples; lanes 1–3), 1.5 (TP, transition-phase samples; lanes 4–6) and 4.0 (SP, stationary-phase samples; lanes 7–9) and run on a 15% SDS-polyacrylamide gel. Lane 10, purified azurin (AZU) 50 ng. The position of azurin is indicated by the arrow. (b) Western blot of whole-cell extracts of PAO1. Lanes 1 and 6, grown aerobically for 16 h in LB. Lanes 2–5, grown anaerobically in LB with 50 mM KNO_{3}. Lane 2, exponential phase; lane 3, late-exponential phase; lane 4, transition phase; lane 5, stationary phase. The last lane contains a sample of purified azurin showing a faint band in the upper part of the gel which is due to azurin dimers. All samples were normalized for the same amount of cells. The upper band in lanes 1–9 in (a) and lanes 1–6 in (b) is a cross-reaction of the antibodies and is probably not related to azurin since it was also observed in extracts of the azurin-deficient strain PSA1 (see Fig. 2b).

Under a variety of conditions, was analysed by Western blotting of whole-cell extracts. The results of samples taken at three representative time-points, mid-exponential phase (ML), transition phase (TP) and stationary phase (SP), from batch cultures growing with good aeration is shown in Fig. 3(a). In the wild-type strain PAO1 (lanes A), little azurin was present in the cell during exponential growth. However, upon transition to the stationary phase, a significant amount of azurin was detected. The high level of azurin expression continued into the stationary phase with little change between samples from the early and late stationary phase. In the anr mutants PAO6261 and S1239 (Fig. 3a, lanes B and C respectively) the azurin concentration also increased in the transition phase but the final amount in the stationary phase was lower than that observed in PAO1. The lower azurin content in stationary-phase cells (i.e. low oxygen pressure in the medium) of the two anr mutants and the presence of an ANR-binding site in the azu promoter region is in agreement with a regulatory role for ANR in azurin expression.

Control of azurin expression by ANR predicts a higher expression level under anaerobic growth conditions. This is shown in Fig. 3(b) for the wild-type strain. Both the basal level of expression, i.e. the amount of azurin observed during exponential growth, and the final level in the stationary phase were higher during anaerobic growth than were the levels observed in an aerobic culture. An increase in anaerobic expression was observed during the transition to stationary phase, indicating that a second regulatory mechanism is in operation. The increase in azurin levels observed in the transition from the exponential to the stationary phase under aerobic conditions may be due to the concerted action of ANR and this second regulatory mechanism.

Predictor activity in PAO1 under aerobic and anaerobic conditions

Upstream of the azu gene two promoters have been identified by S1 mapping (Arvidsson et al., 1989). One of them, P1, is preceded by a sequence, TTGAC-N_{4}-ATCAG, with high similarity to the consensus recognition site of FNR (identical bases are underlined). The distal promoter, P2, has the GG and GC motifs characteristic for promoters recognized by RNA polymerase containing the alternative $\sigma^N$ factor, although the motifs do not have the correct spacing (Fig. 4). The activity of both promoters was assayed in strains harbouring lacZ promoter-probe constructs (Fig. 1a). Total activity was determined with plasmid pGC110 containing both P1 and P2. The individual contributions of the promoters were measured with the constructs pGC115 (P1) and pGC116 (P2).

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P2 AAGCGCTGCGCGGACGTTCGGCTCGACGCGCGAACGCGCCACATCTGTC\_TA\_AAAGCCGAGT 344

P1 GGGTTTGACCTGATCACGTTGGATTGCGGGATCGTTCGCTC\_7\_TTTGCAGATT 458

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Fig. 4. Nucleotides are numbered according to Holtsink et al. (1990). Transcription start sites are at T(344) and T(458). From the data in the paper by Arvidsson et al. (1989) it is difficult to determine the start site of the P1 promoter. The authors indicate position T(457), but the present alignment on the basis of sequence homology shows that T(458) is more likely. The top sequence is the region upstream of the P2 start site and the bottom sequence is upstream of the P1 start site. Identical bases are in bold face. The 'FNR-binding sequence' upstream of the -35 region of P1 is underlined. The GG and GC motifs in the P2 promoter region are in italics and underlined.

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Expression and role of azurin in *P. aeruginosa*

![Graph showing expression of azurin promoters in aerobic and anaerobic conditions.](image)

**Fig. 5.** The activities of the azurin promoters are expressed in β-galactosidase units (kU) and were determined in wild-type *P. aeruginosa* PA01. The promoter-probe plasmids, pGC110 (P1+P2), pGC115 (P1) and pGC116 (P2), contain the azurin promoter(s) upstream of *lacZ* (see Methods). Cells were grown in LB medium (aerobic) or LB medium with 50 mM KNO₃ (anaerobic). Samples were taken at the points (A, B, C, D and E) indicated in the graph of the growth curve (inset).

**Table 3. Promoter activities**

Promoter activities are expressed as β-galactosidase activities (in thousands of Miller units, kU) in cells in the exponential phase (exp) and stationary phase (stat). Aerobic cultures were grown in LB medium (10 ml per 100 ml flask) with vigorous shaking. Anaerobic cultures were grown in LB medium with 50 mM KNO₃ in tightly capped bottles filled to 95% volume. Data are corrected for differences in plasmid copy number. The plasmid copy number in an exponential culture of PA01 harbouring pGC110 is used as a reference. The relative copy numbers were determined by counting the number of transformants obtained in *E. coli* after transformation with plasmid DNA isolated from an equal amount of cells from each of the *P. aeruginosa* cultures used in this study. Little fluctuation has been observed in the copy number of the various constructs. The β-galactosidase values represent the mean of at least three independent experiments. The individual measurements differed by 15% or less. Under all conditions the sum of the measurements of the individual promoters is equal to the value measured for the intact promoter region. Therefore, it is highly unlikely that separation of the two promoters affects the regulation of the individual promoters.

<table>
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<th>Growth conditions</th>
<th>PA01</th>
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<tr>
<td></td>
<td>exp</td>
<td>stat</td>
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<td><strong>Aerobic</strong></td>
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<td>pGC116 (P2)</td>
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</tbody>
</table>

In the wild-type strain PA01 the activity of both promoters in aerobic cultures was strongly stimulated in the transition phase from exponential growth to stationary phase and the highest activity was observed in stationary-phase cells (Fig. 5). Under anaerobic conditions the promoter activity was higher than under aerobic conditions and there was also an increase in activity with the age of the culture. The P1 activity increased more than did the P2 activity (Fig. 5). The higher promoter activity in the exponential growth phase of an anaerobic culture compared to that of an aerobic culture is an indication of transcription induction by anaerobiosis. On the other hand, the increase in promoter activity during the transition from exponential growth to the stationary phase in an anaerobic culture is a measure of induction by a transcription factor related to stationary-phase growth.

**The P1 promoter is regulated by ANR**

The promoter activities were also determined throughout growth in *anr* and *rpoS* mutant strains. In all strains promoter activity was low during exponential growth and higher in the stationary phase (Table 3). In the *anr* mutant PA06261 the profile of P1 and P2 activity during aerobic growth was similar to that shown for PA01 in

2859
Fig. 5. However, the P1 promoter activity in the stationary phase was reduced by almost 40% compared to PAO1 (Table 3). Inoculation of PAO6261 under anaerobic conditions in liquid medium resulted in very slow growth, as can be expected from a strain deficient in one of the major transcription factors involved in anaerobiosis. In accordance with the ANR-binding site upstream of P1, which suggests regulation of P1 by ANR, the P1 activity in PAO6261 did not respond to anaerobic conditions (Table 3). The P2 activity, which increased slightly in PAO1 grown under anaerobic conditions, did not respond to anoxic conditions in PAO6261. Although this promoter does not contain a ANR-binding site, we conclude that the absence of ANR has an effect on its activity. This ANR effect could be indirect and therefore we investigated the promoter activity in a strain deficient in DNR (RM536). This transcription regulator, also belonging to the FNR family, plays a role in switching between aerobic and anaerobic growth conditions. ANR has recently been cloned and sequenced by Tanaka (1994). The expression of the azu operon, encoding azu, was investigated. The rpoS gene of P. aeruginosa, encoding σ5, was recently cloned and sequenced by Tanaka & Takahashi (1994). The expression of rpoS is controlled according to the growth phase and similar to that of azu. In the exponential growth phase the expression of rpoS is low, but during transition to stationary-phase transcription it is strongly induced (Fujita et al., 1994). The activities of the two azu promoters were measured in PSA30, a rpoS mutant constructed by gene replacement which disrupted the coding region of rpoS by insertion of the tetracycline-resistance gene. In stationary-phase cells of aerobic and anaerobic cultures the activity of both promoters was reduced by more than 50% compared to the wild-type strain PAO1 (Table 3). Western-blot analysis of PAO1 and PSA30 samples confirmed the reduction of azurin expression in PSA30. Activation of P1 by ANR was still fully operational in the rpoS mutant (compare P1 activity in aerobic cultures with that in anaerobic cultures in Table 3). The fact that azurin is still expressed in PSA30 indicates that RNA polymerase containing other σ factors can take over in the absence of σ5.

### Table 4. Hydrogen peroxide and paraquat sensitivity of wild-type and Azu- P. aeruginosa

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant phenotype</th>
<th>H2O2</th>
<th>Paraquat</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO1</td>
<td>Wild-type</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>PSA1</td>
<td>Azu-</td>
<td>29</td>
<td>25</td>
</tr>
<tr>
<td>PSA1(pGC135)</td>
<td>Azu-/Azu+</td>
<td>21</td>
<td>19</td>
</tr>
</tbody>
</table>

Azurin is involved in the response to redox stress

The enhancement of azurin expression at the onset of and during the stationary phase indicates that the physiological role of azurin may be found in redox processes related to changes in growth conditions. The transition of a bacterial culture to the stationary phase is dependent on many factors such as nutrient limitation, reduced oxygen pressure and the presence of toxic metabolic end-products. We decided to test whether the response to stress conditions in P. aeruginosa depends on the presence of azurin. The wild-type strain PAO1 and the azu mutant PSA1 were compared for their responses to heat and cold shock, nutritional shift-down, and for their growth on various carbon sources. However, in none of these experiments was a significant difference between PAO1 and PSA1 observed. Only upon challenging both strains with hydrogen peroxide and the superoxide generator paraquat did a significant difference show. The growth-inhibition zones around the disks were taken as a measure of the sensitivity of the strains to the chemicals applied to the disks. This assay clearly demonstrated that the azu mutant was more sensitive to both compounds (Table 4). The twofold increase in sensitivity of PSA1 was also confirmed on gradient plates and in liquid cultures containing paraquat or hydrogen peroxide. To exclude polar effects of the mutation in PSA1, the strain was transformed with pGC135, harbouring and expressing azu, and it was demonstrated that the sensitivity to both paraquat and hydrogen peroxide was restored to wild-type levels (Table 4). The hypothesis that azurin operates in electron transfer mechanisms related to the cellular response to redox stress will be investigated further in future experiments.

### DISCUSSION

Azurin expression analysis by Western blotting showed that production is induced by two distinct growth conditions, the transition from exponential phase to stationary phase and anaerobiosis. It is concluded from these measurements that the trigger for enhanced azurin expressi...
production must be related to the cessation of growth and the limitation of oxygen in the culture. Investigation of the regulation of the two azu promoters showed that the activity of both promoters is dependent on the presence of sigma factor $\sigma^{N}$. In the absence of RNA polymerase containing $\sigma^{N}$ ($\sigma^{E}$) the promoter activity is more than 50% reduced. Despite the low homology between the two promoter regions (Fig. 4), both P1 and P2 seem to be under the control of $\sigma^{N}$. It is tempting to conclude that both promoters are recognized by $\sigma^{E}$, but we cannot exclude the possibility that a transcription factor whose expression is regulated by $\sigma^{E}$ is involved. In addition to control by $\sigma^{N}$, the P1 promoter is also regulated by ANR, a transcription factor which triggers the expression of genes involved in anaerobiosis (Sawyers, 1991; Zimmermann et al., 1991; Winteler & Haas, 1996). Although the P2 promoter region has similarity to promoters recognized by RNA polymerase containing the alternative $\sigma^{N}$ factor, we could not find an effect of NH$_4$Cl, KNO$_3$ or NaNO$_3$ on the overall azu expression or the individual promoter activities (data not shown). Therefore, a regulatory role for these compounds or $\sigma^{N}$ is not likely.

The gene disruption experiments demonstrate that azurin is not an obligatory redox partner of NIR and that azurin does not take over the electron donation from cyt-c$_{551}$ under anaerobic conditions with nitrite as the final electron acceptor. Surprisingly, the cyt-c$_{551}$ mutants PSA15 and PSA16 did grow in liquid anaerobic cultures with nitrite, although slower and to a lower final cell density than did the wild-type strain (Table 2). Zennaro et al. (1993) have shown that a nir$S$ disruption mutant of P. aeruginosa no longer grows anaerobically with nitrate as the acceptor, and De Boer et al. (1994) observed in Paracoccus denitrificans that all nir mutants were severely reduced in anaerobic growth with KNO$_3$. We therefore conclude that in PSA15 and PSA16 either a low level of nitrite, produced by the nitrate reductase, is tolerated, or an alternative and less efficient electron-transport pathway to NIR may be operational.

None of the functions of azurin put forward in the literature (Duine, 1995; Edwards et al., 1995; Hyun & Davidson, 1995) could be confirmed in vivo. The azu mutant did grow on ethanol like the wild-type strain, albeit slowly, and the primary aromatic amine tyramine did serve as a carbon source for both PAO1 and PSA1. Two possibilities that could not be tested because the corresponding electron-transfer pathways are not known to be present in P. aeruginosa are a role for azurin as the electron donor to a copper-containing NIR (Zumft et al., 1987) and as the electron acceptor of methylamine dehydrogenase (Gak et al., 1995). In the light of the observations we described for P. aeruginosa it is unlikely that azurin is needed as the obligatory component in any of the corresponding pathways.

In vitro data show that azurin is capable of interacting with various redox proteins, but the in vivo data suggest that these interactions are not of importance under physiological conditions. The question therefore remains as to the physiological function of azurin. The expression profile of azurin points in the direction of an electron-transfer function during times of stress, such as in transition to the stationary phase. Challenging the wild-type strain PAO1 and the azu-mutant PSA1 with hydrogen peroxide and the oxygen radical generator paraquat in a diffusion assay revealed a much higher sensitivity of the mutant strain. We therefore propose that the physiological role of azurin involves electron transfer directly related to the cellular response to redox stress. The need for an increased expression of azurin in the exponential phase of anaerobically growing cells could be related for instance to the observation made in E. coli that nitric oxide induces the oxidative stress response (Nunoshiba et al., 1993). In P. aeruginosa nitric oxide is produced by nitrite reductase and, to avoid intracellular damage caused by nitric oxide which is not immediately converted to nitrous oxide by nitric oxide reductase, a stress response could be triggered with a concomitant higher expression of azurin.

The cytochrome c peroxidase (CCP) of P. aeruginosa has been shown to interact with azurin in vitro (Foote et al., 1992). Thus, CCP might be one of the physiological partners of azurin. The recent cloning of the CCP gene from P. aeruginosa (Ridout et al., 1995) can be of help in future in vivo and in vitro investigations to identify the true redox partner(s) of azurin.

ACKNOWLEDGEMENTS

We are grateful to the late Professor M. C. Silvestrini for the gift of nitrite reductase serum, and we thank Drs D. Haas and H. Arai for the P. aeruginosa strains, and Drs K. Tanaka and H. Takahashi for the rpoS clone. Critical reading of the manuscript by Drs C. Dennison and M. Ph. Verbeet is gratefully acknowledged.

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Expression and role of azurin in \textit{P. aeruginosa}


Received 19 March 1997; revised 6 June 1997; accepted 10 June 1997.