RESEARCH LETTER

Copper acquisition by the SenC protein regulates aerobic respiration in *Pseudomonas aeruginosa* PAO1

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Abstract

Aerobic respiration of *Pseudomonas aeruginosa* involves four terminal oxidases belonging to the heme–copper family (that is, three cytochrome *c* oxidases and one quinol oxidase) plus one copper-independent, cyanide-insensitive quinol oxidase (CIO). The PA0114 gene encoding an SCO1/SenC-type protein, which is known to be important for copper delivery to cytochrome *c* in yeast, *Rhodobacter* spp. and *Agrobacterium tumefaciens*, was found to be important for copper acquisition and aerobic respiration in *P. aeruginosa*. A PA0114 (*senC*) mutant grew poorly in low-copper media and had low cytochrome cbb_3-type oxidase activity, but expressed CIO at increased levels, by comparison with the wild-type PAO1. Addition of copper reversed these phenotypes, suggesting that periplasmic copper capture by the SenC protein helps *P. aeruginosa* to adapt to copper deprivation.

Introduction

Some natural environments in which the metabolically versatile Gram-negative bacterium *Pseudomonas aeruginosa* thrives contain little bioavailable copper. This trace element is an important cofactor in numerous biological redox reactions, especially in terminal steps of respiration. Factors that can limit Cu availability are pH values > 7.4 and the presence of organic ligands having high affinity for Cu. For instance, in alkaline water and soil, Cu is present as poorly soluble carbonates and hydroxides (Karthikeyan *et al.*, 1997) and forms complexes with humic acids and organic matter (Alloway & Tills, 1984). In blood serum, Cu is firmly bound primarily to three proteins: albumin, ceruloplasmin and transcuprein, resulting in an estimated free Cu^{2+} concentration of about 10^{-13} M (Linder & Hazegh-Azam, 1996).

Pseudomonas aeruginosa has five terminal oxidases for aerobic respiration (Williams *et al.*, 2007). Four of these enzymes – an aa_3 oxidase (*cox*), two cbb_3 oxidases (*cco1* and *cco2*) and a quinol oxidase (*cyo*) – contain copper in their active sites. The fifth enzyme, a cytochrome *bd*-type, cyanide-insensitive quinol oxidase (CIO), does not contain copper (Comolli & Donohue, 2002; Cooper *et al.*, 2003). The cbb_3-type oxidases and CIO have high affinity for oxygen (Alvarez-Ortega & Harwood, 2007). We have shown previously that CIO is able to support aerobic growth of *P. aeruginosa* during severe copper deprivation imposed by a copper chelator (Frangipani *et al.*, 2008). Thus, one strategy that enables *P. aeruginosa* to cope with restricted availability of copper is to use an alternative respiratory pathway that does not require copper. The two major siderophores of *P. aeruginosa*, pyoverdine and pyochelin, both bind Cu^{2+} ions (Braud *et al.*, 2009a, b) and, in theory, might promote copper uptake. It is unlikely, however, that such a mechanism is physiologically relevant, as severe copper limitation results in a strong repression of the pyoverdine and pyochelin biosynthetic genes (Frangipani *et al.*, 2008).

To obtain further insight into the response of *P. aeruginosa* to copper starvation, we mutated the PA0114 gene, which encodes a protein belonging to the SCO1/SenC family. SCO1 (synthesis of cytochrome *c* oxidase) proteins are known to bind copper and to be involved in the assembly of cytochrome *c* oxidases, both in the yeast *Saccharomyces cerevisiae* and in *Rhodobacter* spp. (Krummeck & Rodel, 1990; Nittis *et al.*, 2001; Swem *et al.*, 2005). Members of this family play a role in redox reactions via the reversible oxidation of an active centre disulfide bond. In *S. cerevisiae*, SCO1 delivers copper to the dinuclear CuA site in cytochrome *c* oxidase (Abajian & Rosenzweig, 2006). In *Rhodobacter sphaeroides*, the SCO1 homologue PrrC appears to

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transduce an inhibitory signal derived from the cbb_3 cytochrome oxidase to the PrrBA two-component system, which is homologous to the RoxSR system of *P. aeruginosa*, leading to low expression of photosynthetic genes under aerobic conditions (Eraso & Kaplan, 2000). In *Rhodobacter capsulatus*, PrrC is termed SenC. This protein promotes optimal activity of the cytochrome cbb_3 oxidase by functioning as a copper chaperone, i.e. by sequestering and delivering copper to the active site of this oxidase (Swem *et al*., 2005).

We found that a PA0114 (*senC*) mutant was severely handicapped in a low-copper medium, presumably as a consequence of malfunctioning of the heme–copper terminal oxidases. The *senC* mutant, like a quadruple *cco1,2 cox cyo* mutant, showed upregulated CIO expression in the absence of copper. The SenC phenotypes could be restored to wild-type levels by the addition of copper. These results suggest that SenC participates in copper acquisition, which most probably occurs in the periplasm of *P. aeruginosa*.

Materials and methods

Bacterial strains and culture conditions

Strains and plasmids used in this study are listed in Table 1. All media and solutions were prepared with deionized, double-distilled water. Bacteria were routinely grown on nutrient agar and in nutrient yeast broth (Stanisich & Holloway, 1972) at 37 °C. When required, antibiotics were added to these media at the following concentrations: 12.5 μg mL^{-1} tetracycline, 25 μg mL^{-1} kanamycin for *Escherichia coli* and 100 μg mL^{-1} tetracycline for *P. aeruginosa*. Growth and β-galactosidase experiments were performed in a minimal medium (OS-glucose) containing 0.5% (w/v) glucose, 0.1% (w/v) ammonium sulfate, 0.01% (w/v) Triton X-100 and salt solutions (Ornston & Stanier, 1966) from which $CuSO_4$ was omitted unless stated otherwise. Growth in OS-glucose medium was obtained in 100-mL Erlenmeyer flasks filled with 20 mL of medium, under conditions of good aeration (shaking at 180 r.p.m.) at 37 °C.

Construction of plasmids and gene replacement mutants

DNA cloning and plasmid preparations were performed according to standard methods (Sambrook *et al*., 1989). Large-scale preparations of plasmid DNA were obtained using JETstar 2.0 (Genomed). For inactivation of the PA0114 (*senC*) gene in the *P. aeruginosa* PAO1 chromosome, a 943-bp fragment overlapping the ATG of PA0114 and a 1024-bp fragment overlapping the TGA of PA0114 were amplified by PCR using the primer couples PA0114UPFW: 5′-CCCGGATCCATGG CCACCGTCATCGATC-3′/PA0114UPRV: 5′-CCCGAATTC CATGGGTGGGCAAGGCTC-3′ and PA0114DWFW: 5′-CG CGAATTCGCCTGATTCCCTTTCCTTCC-3′/PA0114DWRV: 5′-GGGAAGCTTCGGAACAGACCAGCACG-3′, respectively. These products were digested with BamHI–EcoRI and EcoRI–

Table 1. Strains and plasmids used in this study

	Relevant characteristics	References or sources
Strains		
E. coli		
DH5α	*recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)U169 [ϕ80d*lacZ*Δ*M15*]F$^-$ Nal^r	Sambrook *et al*. (1989)
HB101	*proA2 hsdS20* (r_b^- m_b^-) *recA13 ara-14 lacY1 galK2 rpsL20 supE44 xyl-5 mtl-1* F$^-$	Sambrook *et al*. (1989)
P. aeruginosa		
PAO1 (ATCC15692)	Wild type	Holloway *et al*. (1979)
PAO6437	*cioAB* mutant	Frangipani *et al*. (2008)
PAO6576	PAO1 containing a 627-bp in-frame deletion in the *senC* (PA0114) gene	This study
PAO6593	*coxBA*-PA0117-*coIII* mutant	Frangipani *et al*. (2008))
PAO6594	*roxSR* mutant	Frangipani *et al*. (2008)
PAO6596	PAO1 containing a deletion in the *cyoABCDE* locus	This study
PAO6597	*cyoABCDE coxBA-PA0117-coIII* mutant	Frangipani *et al*. (2008)
PAO6650	PAO1 containing a *cyo cco1 cco2 cox* deletion	Frangipani *et al*. (2008)
PAO6651	*ccoNOQP1 ccoNOQP2* mutant	This study
PAO6660	PAO6437 containing a 627-bp in-frame deletion in the *senC* (PA0114) gene	This study
PAO6721	PAO6594 containing a 627-bp in-frame deletion in the *senC* (PA0114) gene	This study
Plasmids		
pRK2013	Helper plasmid; Tra$^+$ Km^R	Ditta *et al*. (1985)
pME3087	Suicide vector for allelic replacement; Tc^R; ColE1 replicon	Voisard *et al*. (1994)
pME7554	Plasmid carrying a translational *cioA*′-′*lacZ* fusion; Tc^R	Frangipani *et al*. (2008)
pME9302	Suicide construct used for deletion of the *coxAB*-PA0117-*coIII* locus; Tc^R	Frangipani *et al*. (2008)
pME9303	Suicide construct used for deletion of the *cyoABCDE* locus; Tc^R	Frangipani *et al*. (2008)
pME9304	Suicide construct used for deletion of the PA0114 gene; Tc^R	This study
pME9308	Suicide construct used for deletion of the two adjacent *ccoNOPQ* operons; Tc^R	Frangipani *et al*. (2008)

HindIII, respectively, and cloned into the corresponding sites (sites underlined in primer sequences) of the suicide vector pME3087, yielding plasmid pME9304. Plasmid pME9304, carried by *E. coli* DH5α, was then introduced into *P. aeruginosa* strains PAO1, PAO6437 and PAO6594 by triparental mating, using the helper strain *E. coli* HB101 (pRK2013). Merodiploids were resolved as described previously (Ye *et al.*, 1995). The resulting *P. aeruginosa* strains PAO6576, PAO6660 and PAO6721 each carried an in-frame PA0114 deletion. To construct strains PAO6596 (*cyoABCDE*) and PAO6651 (*ccoNOQP1 ccoNOQP2*) we used the suicide plasmids pME9303 and pME9308, respectively, as described previously (Frangipani *et al.*, 2008). In all mutants used here, the deletions were confirmed by PCR, and PCR fragments were checked by sequencing.

β-Galactosidase assays

β-Galactosidase assays (Miller, 1972) were performed with *P. aeruginosa* cultures grown in OS-glucose medium, with or without 1.5 μM $CuSO_4$. Data are mean values of three independent samples ± SD.

Measurement of cytochrome *c* oxidase activity

Respiratory *c*-type cytochromes allow the artificial electron donor *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD; Fluka), to be oxidized to form a blue indophenol compound (λ_{max} 520 nm). Cytochrome *c* oxidase activity was measured spectrophotometrically in whole cells by following the increase in the $A_{520\,nm}$ at room temperature. Briefly, cells were grown in OS-glucose without added $CuSO_4$ or supplemented with 1.5 μM $CuSO_4$. When cells reached the late exponential growth phase, they were washed twice in 0.9% (w/v) NaCl and adjusted to an $OD_{600} \cong 1$. Then 100 μL (*c.* 10^7 cells) was added to 1.4 mL of 33 mM potassium phosphate buffer (pH 7.0) in a cuvette. The reaction was started by the addition of 5 μL of 0.54 M TMPD. Blanks did not contain cells or TMPD. Cytochrome *c* oxidase activity is expressed as μmol TMPD oxidized min^{-1} per 10^7 cells, using 6.1 as the millimolar extinction coefficient for TMPD (Matsushita *et al.*, 1982). Data are mean values of triplicates ± SD.

Results

PA0114 encodes a homologue of SenC/PrrC in *P. aeruginosa*

The ORF PA0114 encodes a protein of the SCO1/SenC family belonging to the thioredoxin-like proteins. Considering the results obtained with SCO1/SenC in yeast and *R. capsulatus* (Nittis *et al.*, 2001; Swem *et al.*, 2005), we hypothesized that PA0114, referred to as $SenC_{P.a.}$ hereafter, may play a critical role in the adaptation of *P. aeruginosa* to low-copper environments. The $SenC_{P.a.}$ protein shares 33% and 37% amino acid identity with SenC of *R. capsulatus* and PrrC of *R. sphaeroides*, respectively (Fig. 1), including the conserved CxxxC domain and the conserved His-173, which form a copper-binding domain (Eraso & Kaplan, 2000; Nittis *et al.*, 2001; Saenkham *et al.*, 2009). SCO1, PrrC, SenC and $SenC_{P.a.}$ also share one transmembrane domain with the N-terminus located in the cytoplasm and the copper-

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PrrC       ----MTKLYAGVAAAAIAALLAGSAAWVFLGRSEERFAGCG-ANQVAG----GAIGGPFT 51
SenC       -MNVSSKTAALAATAAVVVVVGISAAVTLVPHETDRFAACRKGTGSAS----AQIGGPFT 55
ScoI       NGKVREGSIEFSTGKAIALFLAVGGALSYFFNREKRRLETQKEAEANRGYGKPSLGGPFH 120
PA0114     --MTRVNKTVLVLVALVALVLGLTVHKVLTAQRQADPTVLLDAGIVILP--QTRKVPALE 56
                         :   :                                      :

PrrC       LVDQEGRTVTDREVLAKPSLVYFGYTFCPDVCPFDMARNAQAVDILT-EWGIEVTPVFIS 110
SenC       LISETGATVTDRDVITKPSLVYFGYSYCPDVCPIDSTRNAAAVDLLA-ERGHDVTPVFIS 114
ScoI       LEDMYGNEFTEKNLLGKFSIIYFGFSNCPDICPDELDKLGLWLNTLSSKYGITLQPLFIT 180
PA0114     FTNQDGQAVSTASLKGRWHLLFFGYTFCPDVCPTTLAQLRELQGKLPQEVRDDLQVVFVS 116
           :    *   :   :  :  :::**:: ****:*    :       *  :    :  :*::

PrrC       IDPKRDTPEQLKFFAEAIHPDTIALTGTEAQVKAASQAYKTFYRVQESDDD--YYLIDHS 168
SenC       VDAARDTPPVLTEFTDLMSPKMIGLTGTPEQIDAAVKAYRAYYLIRNPGDP--ATLVDHS 172
ScoI       CDPARDSPAVLKEYLSDFHPSILGLTGTFDEVKNACKKYRVYFSTPPNVKPGQDYLVDHS 240
PA0114     VDPNRDTPQQIKQYLGYFNAGFQGLTGTPENIQKLANAMSIPYIPADTSKP--NYTVDHS 174
            *  **:*  :  :   :      ****  ::    :     :             :***
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Fig. 1. Amino acid sequence alignment of PA0114 ($SenC_{P.a.}$) with its homologues PrrC from *Rhodobacter sphaeroides*, SenC from *Rhodobacter capsulatus* and SCO1 from *Saccharomyces cerevisiae*. The conserved copper-binding domains are highlighted by dashed boxes, whereas the predicted transmembrane domains (obtained using TMPRED; http://www.ch.embnet.org) are shown in boxes with solid lines. *, identity; :, similarity.

binding domain located in the periplasm (Fig. 1) (Eraso & Kaplan, 2000). Contrary to the situation in *Rhodobacter* spp., where the *prrC* (*senC*) gene is part of a *prrBCA* cluster (Eraso & Kaplan, 2000), the *senC*. gene of *P. aeruginosa* PAO1 is located near the gene cluster encoding the COX terminal oxidase (PA0105–PA0108) and adjacent to a gene, PA0113, which is 59% similar to a cytochrome *c* oxidase folding protein annotated in *Synechocystis* sp.

Mutational loss of $SenC_{P.a.}$ impairs growth in a low-copper environment

To test whether the $SenC_{P.a.}$ protein might be involved in the assembly of terminal oxidases in *P. aeruginosa*, we constructed a *senC* in-frame deletion mutant (PAO6576). We reasoned that such a mutant should be impaired in its growth in low-copper media, similar to the behaviour of a *cyo cco1 cco2 cox* quadruple mutant (PAO6650). In a copper-replete environment (1.5 μM $CuSO_4$), strain PAO6576 should grow like the wild type, whereas the growth rate of PAO6650 should remain poor. Results (Fig. 2) confirm this expectation. In OS-glucose medium without added copper, strain PAO6576 (open circles) showed a twofold decrease in growth yield after 8 h of incubation, compared with the wild-type strain PAO1 (open squares). The addition of copper to the medium restored the growth of PAO6576 (filled circles) to the rate of the wild-type PAO1 (filled squares). The same copper treatment did not have any effect on PAO6650 (filled and open triangles), whose growth resembled that of the *senC* mutant grown in low copper. This residual growth is essentially provided by CIO (Frangipani *et al.*, 2008).

To confirm that $SenC_{P.a.}$ plays a role in the function of copper-containing terminal oxidases, we constructed the *senC cioAB* and *senC roxSR* double mutants PAO6660 and PAO6721, respectively. Our hypothesis was that, in a low copper environment, the effect of a *senC* mutation would be stronger in the double mutants than in a wild-type background. Aerobic respiration would be severely compromised in the *senC cioAB* double mutant because all known terminal oxidases would be nonfunctional. The *senC roxSR* double mutant was expected to have a similar phenotype as the RoxSR two-component system is the major positive regulator of CIO (Comolli & Donohue, 2002). Both strains PAO6660 (*senC cioAB*; triangles in Fig. 3a) and PAO6721 (*senC roxSR*; circles in Fig. 3b) showed a dramatic reduction of growth during copper shortage (open symbols), compared with their parental strains PAO6437 (*cioAB*; squares in Fig. 3a) and PAO6594 (*roxSR*; diamonds in Fig. 3b). Addition of 1.5 μM $CuSO_4$ (filled symbols in Fig. 3) fully restored the growth of the double mutants, indicating that the *senC* mutation affects the function of the copper-containing oxidases.

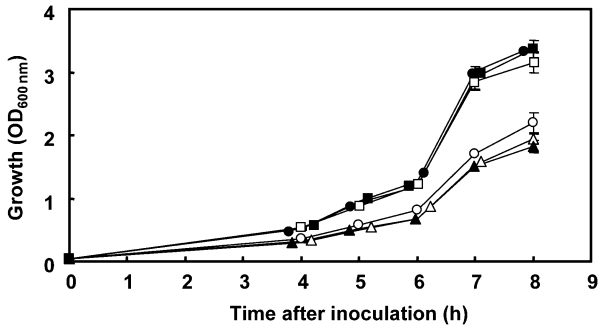

Fig. 2. Growth of the wild-type PAO1 (squares), the quadruple *cyo cco1 cco2 cox* mutant PAO6650 (triangles) and the *senC* mutant PAO6576 (circles). Cells were grown in OS-glucose medium and growth was measured by turbidimetry. Cultures were either not amended with Cu (open symbols) or contained 1.5 μM $CuSO_4$ (filled symbols). Each value is the average of three different cultures ± SD.

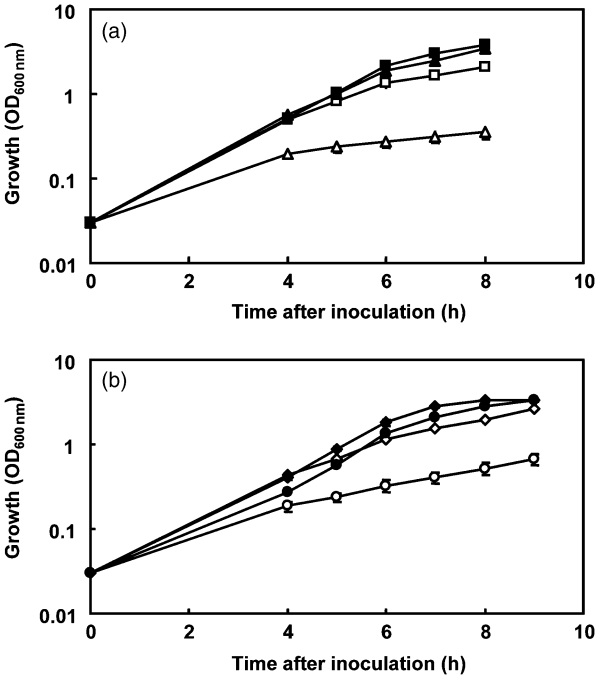

Fig. 3. Effect of a *senC* mutation on different strains during copper shortage. (a) Growth of the *cioAB* mutant PAO6437 (squares) and the *cioAB senC* mutant PAO6660 (triangles). (b) Growth of the *roxSR* mutant PAO6594 (diamonds) and the *roxSR senC* mutant PAO6721 (circles). Cells were grown in OS-glucose medium and growth was measured by turbidimetry. Cultures were not amended with Cu (open symbols) or contained 1.5 μM $CuSO_4$ (filled symbols). Each value is the average of three different cultures ± SD.

TMPD oxidase activity is reduced in a *senC* mutant

The TMPD oxidase test is generally used to measure cytochrome *c* oxidase activity (Comolli & Donohue, 2004).

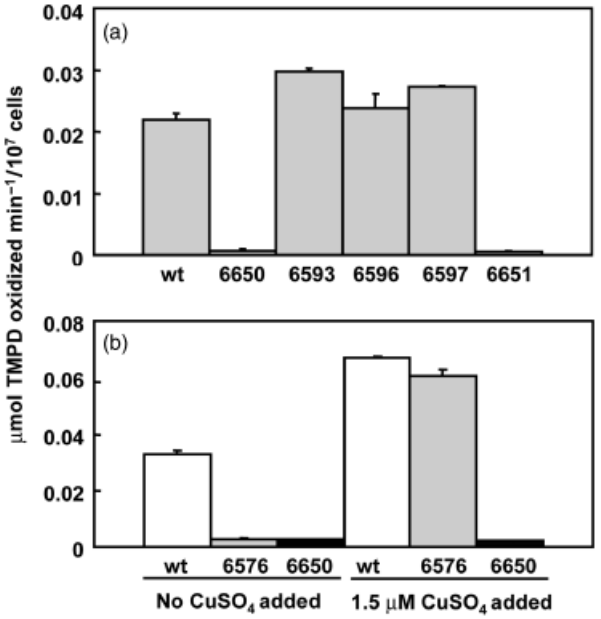

Fig. 4. (a) TMPD oxidase activity of whole cells of *Pseudomonas aeruginosa* PAO1 (wt), a quadruple *cyo cco1 cco2 cox* mutant (PAO6650), a *cox* mutant (PAO6593), a *cyo* mutant (PAO6596), a *cox cyo* double mutant (PAO6597) and a *cco1 cco2* double mutant (PAO6651). (b) Oxidase activity of whole cells of strain PAO1 (wt), the *senC* mutant PAO6576 and the quadruple *cyo cco1 cco2 cox* mutant PAO6650. Cells were grown aerobically in OS-glucose medium without added copper or in the presence of 1.5 µM $CuSO_4$. Activity is expressed as µmol TMPD oxidized min^{-1} per 10^7 cells at pH 7.0 and 25 °C. Each value is the average of three different cultures ± SD.

To investigate which of the four heme–copper terminal oxidases were affected by the *senC* mutation in *P. aeruginosa*, we first assessed which oxidase activity could be detected by the TMPD test under the growth conditions used. To this end, we measured TMPD oxidase activity in the wild type (PAO1), a *cox* mutant (PAO6593), a newly constructed mutant carrying a deletion in the *cyoABCDE* operon (PAO6596), a *cox cyo* double mutant (PAO6597), a newly constructed *cco1 cco2* double mutant lacking both cbb_3-type oxidases (PAO6651) and a quadruple *cyo cco1 cco2 cox* mutant (PAO6650). The *cox* and *cyo* mutations did not affect TMPD oxidase activity, whereas a deletion inactivating the two cbb_3-type terminal oxidases resulted in an almost complete loss of activity, similar to the effect seen for the quadruple mutant (Fig. 4a). The fact that the *cox* mutation had no detectable effect is in agreement with our finding that a *coxB*'-'*lacZ* translational fusion is very poorly expressed under the growth conditions used (data not shown). The strong impact of the *cco1,2* double mutation suggests that oxidation of cytochrome *c* in the TMPD assay is mainly carried out by the two cbb_3-type terminal oxidases, although at this stage we cannot assess the individual contribution of each of the two enzymes. The observation that both cbb_3-type terminal oxidases essentially account for the TMPD oxidase activity measured is consistent with previously published data (Comolli & Donohue, 2004).

To confirm the role of $SenC_{P.a.}$ in the function of terminal oxidases in *P. aeruginosa*, we measured oxidase activity of the wild-type PAO1, the *senC* mutant PAO6576 and the *cyo cco1 cco2 cox* quadruple mutant PAO6650, grown aerobically in OS-glucose medium without added copper or supplemented with 1.5 µM $CuSO_4$ (Fig. 4b). In the absence of added copper, the oxidase activities of both PAO6576 and PAO6650 were very low, by comparison with the wild-type activity. The addition of 1.5 µM $CuSO_4$ resulted in a twofold increase of the oxidase activity in the wild type and functionally complemented the SenC phenotype of strain PAO6576 to the wild-type level (Fig. 4b). As a control, the addition of copper did not affect the oxidase activity of PAO6650 (Fig. 4b). Similar results were also obtained with 20 µM $CuSO_4$ (data not shown). These data confirm that $SenC_{P.a.}$ plays a role in copper acquisition in *P. aeruginosa* and that copper is delivered to the cbb_3-type terminal oxidases and probably to COX as well, although the very low activity of the latter enzyme precludes an assessment.

$SenC_{P.a.}$ regulates CIO expression

We have previously shown that in *P. aeruginosa*, copper represses the expression of the *cioAB* genes in the wild type, but much less so in the quadruple *cyo cco1 cco2 cox* mutant (Frangipani *et al.*, 2008). We therefore hypothesized that a *senC* mutant would show a similar phenotype under low-copper conditions. To test this hypothesis, we monitored the expression of a *cioA*'-'*lacZ* translational fusion, carried by plasmid pME7554, in strains PAO6576 (*senC*) and PAO1 during copper deprivation (Fig. 5). Under these conditions, *cioA*'-'*lacZ* expression in PAO6576 (open triangles) was higher than in the wild type (open diamonds), and this

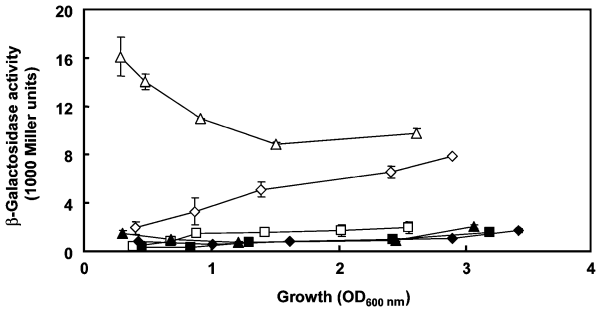

Fig. 5. Expression of a β-galactosidase reporter plasmid containing a *cioA*'-'*lacZ* translational fusion (pME7554) in the wild-type strain PAO1 (diamonds), in a *senC* mutant (PAO6576; triangles) and in a *roxSR* mutant (PAO6594; squares). Cultures were grown in OS-glucose medium and were not amended with copper (open symbols) or contained 1.5 µM $CuSO_4$ (filled symbols). Each value is the average of three different cultures ± SD.

difference was most pronounced at low cell population densities (Fig. 5). When 1.5 μM $CuSO_4$ was added to the growth medium, *cioA*'-'*lacZ* expression was repressed to low levels in both the wild type (filled diamonds) and the *senC* mutant (filled triangles), probably as a consequence of the restored function of the cytochrome *c* oxidases (Fig. 5). As a control, *cioA*'-'*lacZ* expression was measured in the *roxSR* mutant PAO6594 (squares) cultured under the same growth conditions; the expression was low, with and without copper added, as reported previously (Comolli & Donohue, 2002; Frangipani *et al.*, 2008). Taken together, these results show that $SenC_{P.a.}$ has an important impact on the regulation of aerobic respiration in PAO1, in response to copper availability.

Discussion

The results obtained here and in a previous study (Frangipani *et al.*, 2008) indicate that *P. aeruginosa* has at least two different strategies to cope with low copper availability. In the presence of the specific copper chelator bathocuproine disulfonic acid (BCS), a *cioAB* mutant has a strong growth handicap during severe copper deprivation under aerobic conditions (Frangipani *et al.*, 2008), indicating that CIO allows *P. aeruginosa* to respire virtually without copper. This can be considered as a bypass strategy. Here, we used media containing a low copper concentration, but without addition of BCS; the Cu concentration was estimated at around 10^{-8} M by inductively coupled plasma MS (data not shown) and is due to contamination of the chemicals used. Under such conditions, we have found that $SenC_{P.a.}$ insures the delivery of copper to heme–copper terminal oxidases. A *senC* deletion mutant – similar to a *cyo cco1 cco2 cox* quadruple mutant – showed delayed growth during copper limitation, compared with the wild type (Fig. 2). This growth defect could be restored to wild-type levels by the addition of 1.5 μM $CuSO_4$, whereas the quadruple mutant showed the same delayed growth phenotype irrespective of the copper content of the growth medium (Fig. 2). Thus, $SenC_{P.a.}$ insures a second strategy consisting of copper capture from low-copper media.

In the present work, we also show that the *senC cioAB* and *senC roxSR* double mutants, when grown in low-copper media, have the same handicap as that displayed by a single *cioAB* mutant in the presence of BCS. Thus, it appears that the *senC* mutation starves *P. aeruginosa* for copper to the same extent as does a copper chelator. $SenC_{P.a.}$ acts as a copper chaperone delivering copper to the cbb_3-type terminal oxidases (Fig. 4). Whether SenC has the same function in COX and CYO assembly remains to be studied.

The RoxSR two-component system positively regulates CIO expression by transducing and integrating an unknown signal emanating mainly from the cbb_3-1 terminal oxidase (Comolli & Donohue, 2002, 2004). This regulation appears to be particularly important during cyanogenesis under low-oxygen conditions. When endogenously produced cyanide inhibits the heme–copper oxidases, the *cioAB* genes are induced, mainly via the RoxSR system (Comolli & Donohue, 2002; Williams *et al.*, 2007). Moreover, the anaerobic regulator ANR positively regulates the expression of the cbb_3-2 terminal oxidase, but not that of the cbb_3-1 oxidase in poorly aerated cultures (Comolli & Donohue, 2004). ANR also weakly represses the expression of the *cioAB* genes (Cunningham *et al.*, 1997). Our data are compatible with a model according to which *P. aeruginosa* responds to copper limitation under aerobic conditions by upregulating CIO expression. This regulation indirectly involves the copper chaperone $SenC_{P.a.}$.

A recent study (Saenkham *et al.*, 2009), which was published during the preparation of this manuscript, shows that the SenC homologue of *Agrobacterium tumefaciens* delivers Cu ions to the terminal oxidases reacting with TMPD (likely of the aa_3- and cbb_3-types) and that mutations in the conserved Cys71, Cys75 and His163 residues in this Cu chaperone abolish its function. The corresponding Cys84, Cys88 and His173 residues of $SenC_{P.a.}$ (Fig. 1) are therefore likely to form the Cu-binding site in $SenC_{P.a.}$ as well.

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