Developmental defect of cytochrome oxidase mutants of *Streptomyces coelicolor* A3(2)

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To study the link between energy metabolism and secondary metabolism/morphological development in *Streptomyces*, knockout mutants were generated with regard to the subunits of the cytochrome oxidase supercomplex (CcO) in *Streptomyces coelicolor* A3(2). All mutants exhibited an identical phenotype: viable but defective in antibiotic production and cell differentiation when grown in both complex and minimal media. The growth yield of the CcO mutant was about half of that of the WT strain on glucose medium while both strains grew similarly on maltose medium. Intracellular ATP measurement demonstrated that the CcO mutant exhibited high intracellular ATP level. A similar elevation of intracellular ATP level was observed with regard to the WT strain cultured in the presence of BCDA, a copper-chelating agent. Reverse transcriptase PCR analysis demonstrated that the transcription of ATP synthase operon is upregulated in the CcO mutant. Addition of carbonylcyanide m-chlorophenylhydrazone, an inhibitor of ATP synthesis, promoted antibiotic production and aerial mycelia formation in the CcO mutant and BCDA-treated WT cells. We hypothesize that the deficiency of CcO causes accumulation of intracellular ATP, and that the high ATP level inhibits the onset of development in *S. coelicolor*.

**INTRODUCTION**

The soil-dwelling Gram-positive bacterium *Streptomyces* spp. has a complex developmental life cycle resembling that of filamentous fungi. *Streptomyces* form branched, multinucleoid substrate mycelia, which eventually produce aerial mycelia. Aerial mycelia culminate in long spore chains by forming septa at regular intervals (Chandra & Chater, 2013; Chater et al., 2010). *Streptomyces* spp. are also characterized by their ability to produce a wide variety of secondary metabolites that have numerous industrial applications, including antibiotics and anti-tumour activity (Liu et al., 2013; Miyadoh, 1993). Accumulating evidence indicates that the genetic control mechanisms for cell differentiation and secondary metabolite formation are linked to each other and are based on the pleiotropic role of multiple regulatory proteins (Chater et al., 2010).

Recently, we reported the knockout effect of the Sco1/SenC family copper chaperone (ScoC) on cell differentiation and antibiotic production in *Streptomyces coelicolor* A3(2) and *Streptomyces griseus* (Fujimoto et al., 2012). This previous study was performed based on the knowledge that the developmental events in *Streptomyces* spp. are generally stimulated by supplementing copper in the culture medium (Keijser et al., 2000; Kieser & Hopwood, 1991; Ueda et al., 1997). Our results demonstrated that the ScoC protein encoded in the conserved sco (*Streptomyces* copper utilization) operon serves as a global copper chaperone, assisting copper incorporation into diverged copper-dependent oxidases, including cytochrome *c* oxidase and a laccase-like phenol oxidase (Fujimoto et al., 2012). The marked elevation of cytochrome *c* oxidase activity due to the supply of copper raised the possibility that the stimulatory effect of copper on morphological and physiological development is exerted through the promotion of respiration activity (Fujimoto et al., 2012).

Genome information indicates that *S. coelicolor* A3(2) retains two terminal oxidases, cytochrome *bc1*-aa3 supercomplex (referred to as CcO in this paper) and cytochrome *bd* oxidase, which transfer electrons from menaquinol to oxygen (Fig. 1a). CcO exhibits a lower affinity for oxygen and a higher energetic efficiency with respect to proton...
translocation, whereas cytochrome bd oxidase has a higher oxygen affinity and lower energetic efficiency (Bott & Niebisch, 2003). CcO depends on copper whereas cytochrome bd oxidase does not.

Despite the significant role in central metabolism, genetic information concerning the terminal respiratory enzyme with respect to its role is scarce in Streptomyces spp. While it is known that the bd-type quinol oxidase is not essential for the growth of S. coelicolor A3(2) (Brekas & Paget, 2003), the significance of CcO has not yet been evaluated in this organism. In the present study, genes encoding CcO subunits were knocked out, and the effects on the developmental physiology of S. coelicolor A3(2) were assessed. The results show that inactivation of the terminal oxidase inhibits antibiotic production and cell differentiation, and suggest that the developmental defect of the mutants is based on the high intracellular ATP concentration, which negatively influences the initiation of development.

METHODS

Bacterial strains, plasmids and growth conditions. The parental strain S. coelicolor A3(2) M145 (designated WT) and the cosmid clone St078 were obtained from the John Innes Centre, UK. Other cosmid clones including SCOSG4 were generated in our laboratory. The scoC mutant of S. coelicolor was described previously (Fujimoto et al., 2012).

S. griseus IFO13350 was obtained from the Institute for Fermentation, Osaka, Japan. Streptomyces avermitilis MA-4680 was provided by H. Ikeda, Kitasato University. Streptomyces graminis NBRC 15414, Streptomyces flavogriseus NBRC 12771 and Streptomyces venezuelae NBRC 13096 were obtained from the Biological Resource Center, NITE (NBRC), Chiba, Japan. Escherichia coli DH5α strain (Takara Shuzo) was used as a host for conventional DNA manipulation. E. coli strains GM2163 and BW25141/pKD78, used for producing methylation-free DNA and disruption cosmids, respectively, were obtained from the Coli Genetic Stock Center at Yale University. TA cloning of PCR-generated DNA fragments was carried out using pMD19 (Takara Shuzo). pUWLEF carrying an Fpl recombinase gene (Fedoryshyn et al., 2008) was utilized for the construction of a markerless mutant. pKU460, used for genetic complementation, was obtained from Professor H. Ikeda at Kitasato University. The enzymes used for DNA manipulation were purchased from Takara Shuzo. Chemicals were purchased from KOKUSAN, if not indicated otherwise. The standard experimental conditions and materials used for the genetic manipulation of E. coli and Streptomyces strains were as described by Sambrook & Russell (2001) and Kieser et al. (2000), respectively. Streptomyces strains were cultured at 28°C in Bennett’s/sugar medium [composition: 1 g l⁻¹ yeast extract (Difco), 1 g l⁻¹ meat extract (Kyokuto), 2 g l⁻¹ NZ amine (Wako Pure Chemical Industries) and 10 g l⁻¹ of the appropriate sugar (pH 7.2)], R2YE medium (Kieser et al., 2000) and minimal medium [composition: 0.5 g l⁻¹ K₂HPO₄, 0.2 g l⁻¹ MgSO₄.7H₂O, 0.01 g l⁻¹ FeSO₄.7H₂O, 1.0 g l⁻¹ (NH₄)₂SO₄ and 10 g l⁻¹ of the appropriate sugar (pH 7.2)] as described previously (Fujimoto et al., 2012). CuSO₄ (usually at 10 µM) was supplied when required. The copper-specific chelating agent bathocuproinedisulfonic acid (BCDA; Sigma-Aldrich) was added at 400 µM ATP (Sigma-Aldrich) was added at 100 or 500 µM. Solid media were prepared by adding 1.5 % agar to the above mixtures. E. coli strains were cultured in Luria–Bertani (LB) medium [composition: 10 g l⁻¹ tryptone (Difco), 5 g l⁻¹ yeast extract (Difco) and 5 g l⁻¹ NaCl]. E. coli transformants were selected in LB medium containing a final concentration of 50 µg ml⁻¹ ampicillin (Wako), neomycin (Wako) or apramycin (Sigma-Aldrich). Streptomyces transformants were selected in medium containing a final concentration of 20 µg ml⁻¹ kanamycin or 5 µg ml⁻¹ apramycin. The respiration inhibitors carbonyl cyanide m-chlorophenylhydrazone (CCCP) and oligomycin were purchased from Sigma-Aldrich.

Gene deletion. Knockout mutants of S. coelicolor were generated using a homologous recombination technique based on redirect technology (Oust et al., 2003). The cosmid clone SCOSG4 was used for the deletion of ctaCD, qcrCAB, ctaE and ctaA genes. The apramycin-resistant gene cassette constructs used for each disruption were prepared by PCR using the primer sets DistacCD-F/DisctacCD-R (ctaCD), DistqcrCAB-F/DisqtacCAB-R (qcrCAB), DistctacE-F/DisctacE-R (ctaE) and DistctaA-F/DisctaA-R (ctaA) (oligonucleotide primer sequences are summarized in Table 1). The gene cassettes were then substituted for the corresponding coding sequences by in vivo recombination using A RED. The resulting apramycin-resistant cosmids purified from E. coli GM2163 were introduced into the WT strain of S. coelicolor by PEG–protoplast transformation. Apramycin-resistant recombinants were then screened and checked for recombination by PCR using appropriate primer sets.

pKU460-ctaA and pKU460-ctaE, used for genetic complementation, were constructed as follows: DNA fragments containing the coding sequence for ctaA and ctaE were amplified by PCR using the ctaA-F/ctaA-R and ctaE-F/ctaE-E primers (Table 1), recovered as a BglII/HindIII- and HindIII/BamHI-digested fragment and ligated to BamHI/HindIII- and HindIII-digested pKU460 to generate pKU460-ctaA and pKU460-ctaE, respectively. Each plasmid was then introduced into the corresponding mutant by transformation, to generate a kanamycin-resistant transformant carrying the plasmid integrated at the phiC31 site.
A markerless mutant for *ctaCD* was constructed using pUWLFLP, which directs the expression of Flp recombinase (Fedoryshyn et al., 2008). The introduction of this plasmid into the aforementioned apramycin-resistant mutants eliminated the apramycin resistance cassette flanked by flippase recognition target sites.

**Scanning electron microscopy (SEM).** Cells were fixed with 2 % osmium tetroxide for 30 h and then dehydrated by freeze-drying. Each specimen was sputter-coated with palladium/gold using an E-1010 ion sputter (Hitachi) and scanned on a VE8800 scanning electron microscope (Keyence).

**CcO activity measurement.** CcO activity was measured using *N*,*N*,*N*'·tetramethyl-p-phenylenediamine (TMPD) as an electron donor (Frangipani & Haas, 2009; Fujimoto et al., 2012). Using this method, quantification of whole-cell CcO activity as an increment in absorbance (A=520nm) is possible. S. coelicolor strains grown in Bennett's medium/glucose/maltose (liquid/solid) were collected every 24 h for 3 days and washed twice in 0.9 % (w/v) NaCl. Approximately 5 mg cells were added with 1.4 ml 33 mM potassium phosphate buffer (pH 7.0) into a cuvette. The reaction was initiated by adding 5 µl 0.54 M TMPD. CcO activity was expressed as TMPD (nmol) oxidized min⁻¹ (mg cells)⁻¹, with millimolar extinction coefficient as 6.1 for TMPD (Meng et al., 2011).

**Intracellular ATP measurement.** Intracellular ATP was quantified using luciferase (Matsushita et al., 1982). Cells were grown on cellophane-topped agar medium at 28 °C. Approximately 0.1 g (wt weight) cells collected from the surface of the medium was suspended in 0.1 % trichloracetic acid and disrupted by vigorous agitation using a vortex mixer. The mixture was then centrifuged at 18,000 *g* for 10 min. The resultant supernatant was appropriated diluted with 5 mM Tris-acetate (pH 7.8). Subsequently, 100 µl of the diluted solution was added to 50 ml luciferase solution (Lucifer 250; Kikkoman), and the intensity of light emission second⁻¹ was measured using a Synergy 2 luminometer (BioTek) within 1–5 min after initiating the enzyme reaction. Quantification of ATP ranging from 20 fmol to 200 nmol is possible using this method.

**Transcriptional analysis.** *S. coelicolor* cells (approximately 0.1 g) grown on cellophane-covered Bennett's/glucose agar medium were collected and suspended in 1 ml RNAprotect bacteria reagent (Qiagen). Total RNA was extracted using an RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. Methods and conditions for quantitative reverse transcriptase-PCR (RT-PCR) were as described previously (Watsui et al., 2014). Oligonucleotide sequences of primer sets (*atpI*-*, rt-F/*rt-R and *hrdB*-*, F/*rt-R) are summarized in Table 1. The mixture was then centrifuged at 18,000 *g* for 10 min. The resultant supernatant was appropriately diluted with 5 mM Tris-acetate (pH 7.8). Subsequently, 100 µl of the diluted solution was added to 50 ml luciferase solution (Lucifer 250; Kikkoman), and the intensity of light emission second⁻¹ was measured using a Synergy 2 luminometer (BioTek) within 1–5 min after initiating the enzyme reaction. Quantification of ATP ranging from 20 fmol to 200 nmol is possible using this method.

**Table 1. Oligonucleotide primers**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’–3’)*</th>
<th>Restriction enzyme†</th>
<th>Position (nt)‡</th>
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<tbody>
<tr>
<td><em>ctaA</em>-F</td>
<td>CTCGAGAGATTCCTCGCGTGGAGTAAGAACATG</td>
<td>BgIII</td>
<td>6605622–660560</td>
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<tr>
<td><em>ctaA</em>-R</td>
<td>CTCGAGAGATTCCTCGCGTGGAGTAAGAACATG</td>
<td>HindIII</td>
<td>6604444–6604461</td>
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<tr>
<td><em>ctaE</em>-F</td>
<td>CTCGAGAGATTCCTCGCGTGGAGTAAGAACATG</td>
<td>HindIII</td>
<td>6603019–6603036</td>
</tr>
<tr>
<td><em>ctaE</em>-R</td>
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<td>HindIII</td>
<td>6603954–6603937</td>
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<tr>
<td><em>atpI</em>-F</td>
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<td>2830551–2830532</td>
</tr>
<tr>
<td><em>atpI</em>-R</td>
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<td>–</td>
<td>2830433–2830452</td>
</tr>
<tr>
<td><em>atpII</em>-F</td>
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<td>–</td>
<td>2826892–2826873</td>
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<tr>
<td><em>atpII</em>-R</td>
<td>CGATCGAGACCGCGTCAGACG</td>
<td>–</td>
<td>2826714–2826733</td>
</tr>
<tr>
<td><em>hrdB</em>-F</td>
<td>GAAGTATCGAGATCGAGACGA</td>
<td>–</td>
<td>2298671–2298652</td>
</tr>
<tr>
<td><em>hrdB</em>-R</td>
<td>CTCGATGAGGTCGACAGAAGA</td>
<td>–</td>
<td>2298575–2298594</td>
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</tbody>
</table>

*Sequences corresponding to the apramycin resistance gene are shown in lower case.
†Recognition sites are underlined in the previous column.
‡Corresponding position in the genome sequence database of *S. coelicolor*, *S. griseus* and *S. avermectins* (http://gib.genes.nig.ac.jp/).
RESULTS

Generation of knockout mutants

Table 2 summarizes the genes encoding the components of the CcO supercomplex in *S. coelicolor* A3(2). The macromolecule consists of two complexes, bc1 complex and aa3 complex. The former complex contains three proteins, QcrA, QcrB and QcrC (products of SCO2149, 2148 and 2150, respectively), and the latter CtaC (subunit II), CtaD (subunit I), CtaE (subunit III) and CtaF (subunit IV) (products of SCO2156, 2155, 2151 and 2154, respectively) (Bott & Niebisch, 2003; Niebisch & Bott, 2003). *S. coelicolor* retains a CtaD and CtaF paralogue encoded by SCO7234 and 7235, respectively. CtaA and CtaB (encoded by SCO1930 and 1934, respectively) are involved in the biosynthesis of heme incorporated into CcO (Svensson et al., 1993; Svensson & Hederstedt, 1994). Localization of each coding sequence is illustrated in Fig. 1b.

We generated apramycin-resistant knockout mutants with respect to *ctaA*, *ctaE*, *ctaC-ctaD* and *qcrC-qcrA-qcrB*, encoding CcO-related proteins, by using a homologous recombination technique (see Methods). We also generated a markerless null mutant for *ctaC-ctaD* (hereafter, *ctaCD* mutant) encoding the copper- and haem-dependent central oxidase domains of CcO. CcO activity measurement demonstrated that the TMPD-oxidizing activity of the CcO mutants was below detectable level under any condition (see Methods) while the activity of the WT was at the same level as described in a previous report (Fujimoto et al., 2012). This indicates that the knockout was successful. All CcO mutants exhibited identical features.

Growth profiles

The comparison of growth profiles in Bennett’s liquid medium containing glucose or maltose at 1% is represented in Fig. 2. In glucose-containing medium, the cell yield of the mutant was about half of that obtained with the WT strain. The pH of the mutant culture decreased to 4.0 (Fig. 2, left upper panel). In maltose medium, the growth yield of the mutant was almost the same as that of the WT. The mutant did not cause a decrease in pH when cultured in maltose medium (Fig. 2, right). The measurement of culture pH was carried out in order to assess whether the alteration in medium pH is correlated with the mutant phenotype; the normal pH in maltose medium excludes the possibility that the change of culture pH is the direct reason for the mutant phenotype described below.

The growth of the *ctaCD* mutant on minimal agar medium is shown in Fig. 3. In a synthetic medium containing glucose as a carbon source, the mutant was able to produce substrate mycelium to the same extent as the WT strain. The mutant grew even on the medium without sugar. This is probably due to the agar-accumulating activity of this bacterium (Chi et al., 2012). In contrast, the growth of the mutant was significantly inhibited in the maltose-supplemented medium. Similar inhibition was observed with regard to other disaccharides, comprising lactose, trehalose and sucrose, supplied at 1% (data not shown). The mutant grew normally when both maltose and glucose were supplied (Fig. 3). The growth defect on the maltose-supplemented medium could be due to the repression of agar-assimilation activity (see Discussion).

Developmental phenotypes

The developmental phenotypes were observed by growing the strains on Bennett’s agar medium (Fig. 4a). The WT strain of *S. coelicolor* A3(2) grown on the medium supplied with glucose or maltose produced the pigment antibiotics actinorhodin (diffusible purple pigment) and undecylprodigiosin (intracellular red pigment). The WT formed aerial mycelia and spores. SEM observation (Fig. 4b) demonstrated that spore chains were formed after 5 days of growth on glucose medium. In contrast, the *ctaCD* mutant (Fig. S1, available in the online Supplementary Material) and the *ctaA* mutant (Fig. 4) formed substrate mycelia but were defective in producing pigment antibiotics and forming aerial mycelia and spores on Bennett’s/glucose medium. The mutant formed aerial mycelia on Bennett’s/maltose medium. The developmental phenotype did not change even after long-term (~2 weeks or more) incubation. The mutants exhibited the same phenotype on Bennett’s agar containing TES buffer with pH adjusted to 7.2 (data not shown), which verified that the low pH level observed with regard to the mutant growth in glucose medium.

<table>
<thead>
<tr>
<th>SCO no.</th>
<th>Gene</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>SCO2157</td>
<td>ctaC</td>
<td>Cytochrome c oxidase subunit II</td>
</tr>
<tr>
<td>SCO2155</td>
<td>ctaD</td>
<td>Cytochrome c oxidase subunit I</td>
</tr>
<tr>
<td>SCO2154</td>
<td>ctaF</td>
<td>Cytochrome c oxidase subunit IV</td>
</tr>
<tr>
<td>SCO2153</td>
<td>ykuD</td>
<td>l, d-Transpeptidase</td>
</tr>
<tr>
<td>SCO2152</td>
<td>Two-component system response regulator</td>
<td></td>
</tr>
<tr>
<td>SCO2151</td>
<td>qcrC</td>
<td>Cytochrome c heme-binding subunit</td>
</tr>
<tr>
<td>SCO2149</td>
<td>qcrA</td>
<td>Rieske iron-sulfur protein</td>
</tr>
<tr>
<td>SCO2148</td>
<td>qcrB</td>
<td>Cytochrome b subunit</td>
</tr>
<tr>
<td>SCO2147</td>
<td>trpD</td>
<td>Anthranilate phosphoribosyltransferase</td>
</tr>
<tr>
<td>SCO1934</td>
<td>ctaB</td>
<td>Heme O biosynthesis protein</td>
</tr>
<tr>
<td>SCO1930</td>
<td>ctaA</td>
<td>Heme A biosynthesis protein</td>
</tr>
<tr>
<td>SCO7234</td>
<td>ctaD2</td>
<td>Cytochrome c oxidase subunit I</td>
</tr>
<tr>
<td>SCO7235</td>
<td>ctaF2</td>
<td>Cytochrome c oxidase subunit IV</td>
</tr>
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</table>
(Fig. 2) is not correlated with the developmental defect of the mutant.

The mutant phenotype was restored to that of WT when the intact copy of the disrupted gene was introduced into the genome of ctaA (Fig. 4a) and ctaE (data not shown) mutants using a phage-derived integration vector. However, we failed to introduce the intact genes into the mutants for ctaCD and qcr. The introduction was also unsuccessful when we used various plasmid constructions including the corresponding genes as well as their downstream regions. Genetic complementation of the ctaA mutant also verified that the introduction of the intact gene restores CcO activity to the mutant (Fig. S2).

**Intracellular ATP levels**

Furthermore, to assess the energetic state of the respiratory mutant cells, intracellular ATP concentration was measured (see Methods). Interestingly, it was found that the CcO mutants contained a high level of ATP (Figs 5a and S2). A similar feature was observed with regard to the mutant for ScoC, a copper chaperone described in a previous report (Fujimoto et al., 2012). Precise levels fluctuated, but overall the mutants exhibited a three- to fivefold increase in ATP over that of the WT in repeated measurements. The high intracellular ATP level of the ctaA mutant was restored to the WT level by genetic complementation (Fig. S2). Furthermore, the ATP content of WT cells grown in the
presence of Cu$^{2+}$ and BCDA, a copper-chelating agent, was also evaluated. The result (Fig. 5b) showed that the intracellular ATP level was reduced by the addition of 10 µM CuSO$_4$ and was markedly elevated by the addition of 400 µM BCDA. This finding supports the negative correlation between CcO activity and the intracellular ATP level.

**Effect of ATP synthesis inhibitors**

The above-mentioned correlation between the developmental defect and a high intracellular ATP content in the respiratory mutants prompted us to study the effect of ATP synthesis inhibitors. Fig. 6(a) shows the effect of exogenously supplied CCCP, an effective uncoupling agent. The agent inhibited the growth at a high concentration. On the other hand, the addition of this inhibitor at lower concentrations affected developmental growth of the WT strain; it caused the formation of multiple doughnut rings, where different modes of inhibition of pigment production and/or aerial mycelium formation take place. Further, it dramatically promoted the production of pigmented antibiotics and aerial mycelium formation in the ctaCD mutant (Fig. 6a, bottom). Similarly, pigment production and aerial mycelium formation inhibited by the supply of BCDA in the WT strain were restored by the addition of CCCP (Fig. 6b). The addition of oligomycin, an inhibitor of ATP synthase, stimulated pigment production in the WT grown on glucose medium and in the CcO mutant grown on maltose medium (results are shown in Fig. S3).

We also observed the effect of exogenous ATP (Fig. 7). Antibiopic production and aerial mycelium formation of *S. coelicolor* were dramatically inhibited by the addition of 100 or 500 µM ATP. Aerial growth of other *Streptomyces* spp. was also inhibited by the supply of ATP (Fig. 7). These results support the view that intracellular ATP level is critical for the developmental decision and that the high levels of ATP correlate with the developmental deficiency of the respiratory mutant.

**Transcription of atp operon**

To study the basis of the high ATP content, transcriptional analysis was carried out with respect to *atpI* (SCO5366), the first coding sequence of the *atp* operon (SCO5366–SCO5374), encoding the ATP synthase subunits of *S. coelicolor* A3(2). Quantitative RT-PCR using the partial coding sequence of *atpI* revealed that the *atp* operon is expressed in a distinctive manner in the ctaCD mutant, reaching two- to ninefold that of the WT level during the 72 h of culture (Fig. 8a). Similarly, WT cells cultured in the medium supplied with BCDA exhibited high transcription levels (Fig. 8b). In addition, the addition of copper to the medium repressed the transcription at 72 h. These results suggest that the transcription of the *atp* operon is under...
homeostatic control that compensates for the respiratory deficiency.

**DISCUSSION**

This study revealed the knockout effect of CcO on the physiology of *S. coelicolor A3(2)*. It is generally perceived that the development of *Streptomyces* spp. depends on an aerobic environment; however, we were able to generate mutants for CcO in *S. coelicolor A3(2)*. All CcO mutants obtained in this study exhibited the same phenotype. Although we could not obtain genetically complemented strains with regard to the *ctaCD* and *qcr* mutants, the successful complementation of the *ctaA* and *ctaE* mutants indicates that the phenotype of the *cta* mutants obtained in this study is due to the loss of CcO activity. We do not know the exact reason for the failure in the introduction of the DNA fragment containing *ctaCD* and *qcr* into the corresponding mutant, but one possible explanation is that the introduction of the gene encoding the central domains of the respiratory apparatus causes a toxic effect to the cell. It is possible that the knockout construction causes a polar effect on the expression of the downstream coding sequences of the *ctaCD* and *qcr* operons, and that the polarity is fundamental to the mutant phenotypes. However, currently we are not able to verify this possibility owing to the failure to introduce the intact coding sequences into the mutants.

The successful knockout of CcO depending on a high O₂ concentration indicates that the organism can grow even without the respiratory enzyme activity of low affinity for oxygen. This indicates that the high oxygen level is not a critical factor for the growth of *S. coelicolor A3(2)*. Sustainability of *Streptomyces* cells under anoxic conditions has been demonstrated in several studies. van Keulen *et al.* (2003, 2007) reported that *S. coelicolor A3(2)* grows even in anaerobic standing culture and that it maintains viability over several weeks of strict anaerobiosis. Fischer *et al.* (2010) have characterized the three active nitrate reductases retained by *S. coelicolor A3(2)* and showed that one of the enzymes is specifically induced by anoxic conditions. The soil environment, which forms the natural niche of *Streptomyces*, contains relatively low O₂. Hence, the ability to grow under microaerobic conditions should be fundamental to the survival of this microorganism.

The measurement of culture pH demonstrated that the growth of the *ctaCD* mutant causes acidification of the culture medium containing glucose as a carbon source (Fig. 2). This raises the possibility that glucose-dependent growth under respiratory deficiency is based on energy derived from fermentation. In contrast, the normal pH in the culture using Bennett’s/maltose (Fig. 2) and the growth inhibition on minimal agar (Fig. 3) infer that the assimilation of these substrates is dependent only on respiration. Although accumulation of organic acids in *Streptomyces* culture has been observed (Madden *et al.*, 1996), the possibility of their occurrence as fermentation products has not yet been fully studied. The use of respiratory mutants may help in elucidating the details of the alternative energy metabolism in *Streptomyces*.

The CcO mutant was viable on all media tested in this study except that growth was significantly inhibited when

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**Fig. 5.** Intracellular ATP concentration. (a) Intracellular ATP content of *S. coelicolor A3(2)* strains. The WT strain (filled circles), *ctaCD* mutant (filled triangles) and *scoC* mutant (filled diamonds) were grown for 24, 48 and 72 h in Bennett’s/glucose solid medium. (b) Intracellular ATP content of the WT cells grown for 24, 48 and 72 h in Bennett’s/glucose solid medium supplied with neither CuSO₄ nor BCDA (open circles), 10 µM CuSO₄ (open triangles) or 400 µM BCDA (open squares). Data presented are the means of duplicated measurements. The error bars indicate upper and lower values.
minimal agar containing maltose or other disaccharides was used (Fig. 3). At present, we speculate that this growth defect is due to the inability of the mutant to assimilate these sugars. As mentioned above, the organism may assimilate these sugars only by respiration, not by fermentation. The growth defect should be also based on the inactivation of agarase, which is fundamental to the growth of bacteria on minimal agar medium that does not contain any other carbon source (Fig. 3, left). The presence of maltose and other disaccharides exerting the same effect may influence the genetic control of agarase expression.

The growth yield of the WT strain was higher than that of the CcO mutant when Bennett’s/glucose liquid medium was used for cultivation (Fig. 2). Similarly, the WT grew better on agar medium with the same composition (Fig. 4a). Meanwhile, the WT growth on minimal agar medium containing glucose as the sole carbon source was at a similar level to that of the mutant (Fig. 3). This implies that glucose assimilation under CcO-mediated aerobic respiration depends on nutritional conditions. Availability of amino acids and/or vitamins could be a crucial factor fundamental to efficient glucose assimilation. Detailed characterization of the correlation between metabolic and respiratory efficiency is important in order to understand the physiology of the industrial organism.

A notable observation was that the CcO mutant was unable to perform cellular development and antibiotic production. Blundell et al. (2013) reported that knockout of the genes encoding CcO subunits does not affect cell morphogenesis in Streptomyces lividans, a close relative of S. coelicolor A3(2). Despite this, the knockout mutant for Sco2 (the ScoC equivalent of S. lividans) exhibits a distinctive developmental defect. Hence, the researchers predicted the presence of another Cu2+- and Sco-dependent mechanism involved in the initiation of cell differentiation. Recently, Blundell et al. (2014) reported that the radical, copper oxidase GlxA, is associated with the mechanism and plays an essential role in hyphal development in S. lividans. Currently, we do not have any clear explanation for the divergence of the CcO mutant phenotype between the two closely related Streptomyces spp. There may be a back-up mechanism specific to S. lividans. Alternatively, there could be a difference in the mode of homeostatic response to respiration deficiency (see below) and its effect on the cell physiology between the two species.
A simple explanation for the developmental defect of the CcO mutant of *S. coelicolor* A3(2) is that the mutant cannot yield enough energy to allow development. However, the intracellular ATP level in the respiratory mutant was somewhat higher than that in the WT (Fig. 5). Similar elevation of ATP concentration was observed in the WT cells treated with BCDA. Furthermore, the supply of ATP synthesis inhibitors promoted aerial mycelium formation and antibiotic production in the CcO mutant and the BCDA-treated WT (Fig. 6). This intriguing fact in turn infers the presence of a regulatory mechanism that senses the energetic state and transmits the information to the developmental decision process.

Currently, we assume that the high intracellular ATP level in the mutant cell is based on the homeostatic responses to the respiratory defect involving the activity of an alternative respiratory enzyme as well as global gear changes in primary metabolism. Another possible explanation is that the defect in performing secondary metabolism and aerial mycelium development saves ATP. However, our preliminary observation indicated that some of the *bld* mutants of *S. coelicolor* A3(2) defective in both antibiotic production and aerial mycelium formation exhibit a high intracellular ATP level and others do not (our unpublished observation). This excludes the possibility that the high ATP level is simply due to the lack of its use in developmental processes.

The results of RT-PCR analysis (Fig. 8) demonstrated that the transcription level of *atpI*, the first coding sequence of the *atp* operon encoding the subunits of ATP synthase, is significantly upregulated in the respiratory mutant and the WT cultured in the presence of BCDA. We do not yet know whether the overexpression of ATP synthase directly causes a high ATP yield. However, it is possible that the high expression level of the enzyme is fundamental to the high intracellular ATP content. The overexpression of the enzyme may compensate for the respiratory deficiency and maintain the efficiency in energy yield. Presumably, the high intracellular ATP level is also based on the reduction of its consumption rate. Detailed characterization of cellular energetic state is required in order to understand the reason for the alteration in ATP level.

We hypothesize that the high intracellular ATP level becomes a factor fundamental to the developmental defect in the respiratory mutant. A similar idea has been previously proposed by Suh and colleagues, who observed that exogenous supply of a high concentration of ATP inhibits antibiotic production in several *Streptomyces* spp. (Li et al., 2008, 2011; Meng et al., 2011). We confirmed the same effect of exogenous ATP on pigment production and aerial mycelium formation in *S. coelicolor* A3(2) as well as on the morphological development of other *Streptomyces* spp. (Fig. 7). Together with these previously published works, the results of this study, including the effect of ATP synthesis inhibitors, support the notion that a high intracellular ATP level serves as a negative factor critical for the initiation of development.

Respiration efficiency is an important factor in terms of industrial fermentation and production. Generally, it is known that aeration efficiency is critical to the production efficiency of *Streptomyces* spp.; hence, the culture is strongly
agitated. On the other hand, the evidence obtained in this study raises the possibility that inefficiency in respiration is not always fundamental to the reduction of production rate. A thorough understanding of the correlation between the energetic yield and signalling for the developmental decision will help in developing an effective fermentation system for using Streptomyces spp. and related bacteria.

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