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Cytochrome *c*-551 from the thermophilic bacterium PS3 grown under air-limited conditions

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A small-sized *c*-type cytochrome, designated cytochrome *c*-551, was prepared from membrane fraction of the thermophilic bacterium PS3, grown under air-limited conditions by extraction with cholate, precipitation with polyethylene glycol, and successive chromatographies with DEAE-cellulose and Sephacryl S-200 in the presence of a detergent. The purified sample contained approx. 1 mol of heme *c* per 10000 g protein; it showed absorption bands at 551, 522 and 416 nm upon reduction, and a Soret peak at 409 nm upon oxidation. This cytochrome showed a single band of 10 kDa on polyacrylamide gel electrophoresis with sodium dodecyl sulfate. The isoelectric point of this cytochrome *c*-551 was pH 4.0. Cytochrome *c*-551 was suggested to play an important role in the respiratory chain with a terminal oxidase cytochrome *o*, which is produced under air-limited conditions, since cytochrome *c*-551 could mediate electron transfer between cytochrome $bc_1(b_6 f)$ complex and cytochrome *o*, showing quinol oxidase activity.

Introduction

The Gram-positive, spore-forming thermophilic bacterium PS3 contains cytochrome *o* as well as cytochrome aa_3 as terminal oxidases [1]. When these cells are cultured under highly aerobic conditions, their terminal oxidase is an aa_3-type containing covalently-bound cytochrome *c* [2,3], whereas it is an *o*-type oxidase when they are cultured under air-limited conditions, where the growth rate is proportional to the oxygen concentration [4]. It is also known that the caa_3-type oxidase forms a super complex with the cytochrome $bc_1(b_6f)$ complex in PS3 cells grown under highly aerated conditions, without requiring another cytochrome *c* [5]. In contrast, PS3 cells grown under air-limited conditions synthesized higher amounts of *b*- and *c*-type cytochromes, suggesting that a different respiratory chain is operating in these cells. Although parts of these *b*- and *c*-type cytochromes are explained by the presence of cytochrome *o* and the cytochrome bc_1 complex, the major difference from the respiratory chain under aerobic conditions is likely due to the presence of a cholate-extractable *c*-type cytochromes. Here we report the purification and properties of this cytochrome *c*-551, which links the bc_1 complex and cytochrome *o* in the respiratory chain of PS3 cells grown under air-limited conditions. To the best of our knowledge, this is the first report on the small-sized membrane-bound *c*-type cytochrome from a Gram-positive bacterium, whose role in the respiratory chain is defined.

Experimental procedures

Materials

The thermophilic bacterium PS3 was cultured in a medium containing 0.8% polypeptone, 0.4% yeast extract and 0.3% NaCl at 66–69°C under air-limited conditions as described previously [1]. The methods for preparing membranes and cytochrome *o* from the air-limited cells were the same as described previously [4]. PS3 cytochrome bc_1 complex was prepared as described from sufficiently aerated cells [6]. The low-molecular-weight protein standards (bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme) were purchased from Bio-Rad, Richmond, CA. Horse-heart cytochrome *c* and Mops were purchased from Sigma, St. Louis, MO. DEAE-cellulose (DE-52) and pancreatic trypsin inhibitor were products of Whatman (U.K.) and Worthington Bio-

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Abbreviations: Mops, 4-morpholinepropanesulfonic acid; SDS, sodium dodecyl sulfate.

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chemicals, respectively. Other reagents were obtained as described previously [4,6].

Measurement of oxidase activity

Cytochrome *c* oxidase activity was followed with a pH meter (Beckman 4500) with ascorbate (10 mM) in a medium (1.6 ml) composed of 25 mM K_2SO_4, 2.5 mM $MgSO_4$ and 1 mM K · Mops (pH 6.4) at 40 °C as described previously [7]. Ubiquinol oxidase activity was followed with an oxygen electrode (YSI No. 4001) in a semi-closed cell (3 ml) in the presence of malate dehydrogenase and malate for re-reduction of ubiquinol at 40 °C, essentially as described previously [5,6]. The reaction medium was composed of 20 mM sodium phosphate buffer (pH 6.8), 1 mM EDTA, 20 μg/ml of sonicated PS3 phospholipids, 30 μM ubiquinol-1, 5 mM DL-malate and PS3 malate dehydrogenase (0.1 units/ml).

Pyridine hemochrome and spectroscopy

Heme *c* content in the final preparation was determined after converting them to pyridine ferrohemochrome with 0.05–0.1 M NaOH, 10% (v/v) pyridine and a small amount of Na_2SO_4. A molar absorption coefficient of 24.3 $mM^{-1} \cdot cm^{-1}$ was used to determine the absorbance difference between the reduced form and the oxidized form at 550–535 nm [8].

The redox potential of cytochrome *c*-551 was spectrophitometrically determined by adding known amounts of ferrocyanide and ferricyanide.

Absorption spectra were measured with a recording spectrophotometer (Shimadzu UV-240) at room temperature. The spectrum at liquid nitrogen temperature was obtained using a Union-giken spectrophotometer (US-401) with a low-temperature measurement attachment. A cell with a 3 mm light path was used.

High-performance gel-filtration chromatography

A TSK G3000 SW column (Toso Co., Tokyo) was used with 20 mM Mops buffer (pH 6.8)/0.4% cholate/0.5 M NaCl/1 mM EDTA.

Other methods

SDS-polyacrylamide gel electrophoresis was carried out according to the method of Schägger et al. with a 10% gel [9]. Staining for hemes was carried out with *o*-tolidine [10].

Other methods were the same as described previously [5,6].

Purification of PS3 cytochrome c-551

Step 1. Washed membranes from air-limited PS3 cells (red membranes) were suspended in a solution containing 2% cholate, 0.1 M Na_2SO_4, 30 mM Tris-H_2SO_4 buffer (pH 8.0) and 1 mM EDTA in a final volume of 100 ml. The mixture was sonicated in an ice bath for 10 min, stirred for 30 min, and then centrifuged at 140000 × *g* for 40 min.

Step 2. Poly(ethylene glycol) 6000 was added to the supernatant fraction of Step 1 at a final concentration of 8%. The slightly turbid solution was stirred for 30 min at room temperature and then centrifuged for 20 min at 32000 × *g*. The pink supernatant was mixed with poly(ethylene glycol) and $MgSO_4$ at a final concentration of 30% and 5 mM, respectively, and stirred and centrifuged as before, and the red precipitate was solubilized in 30 mM Tris-H_2SO_4 buffer (the buffer) containing 0.5% Triton X-100.

Step 3. The sample from Step 2 was applied to a DEAE-cellulose column (2.5 × 8 cm). The column was then washed with the buffer containing 0.5% Triton X-100 and 5 mM NaCl (200 ml). Upon raising the NaCl concentration to 15 mM, the red band due to cytochrome *c*-551 moved slowly, and when the band was 1 cm from the bottom it was eluted by raising the NaCl concentration to 30 mM. The red peak fraction (about 70 ml) was collected.

Step 4. This fraction was diluted with water to a conductance of 0.2 $m\Omega^{-1} \cdot cm^{-1}$ and then applied to a DEAE-cellulose column (1.5 × 13) cm. The column was eluted as in Step 3.

Step 5. Ammonium sulfate was added to the eluate from Step 4 to give 50% saturation and the solution was centrifuged for 5 min at 10000 × *g*. The red floating pellet was saved and diluted with the buffer containing 0.4% cholate (2 ml). It was then mixed with Bio-Beads SM-2 to remove excess Triton X-100. The soluble fraction was applied to a Sephacryl S-200 (Pharmacia) column (1.5 × 90 cm) equilibrated with 20 mM Mops buffer (pH 6.8) containing 0.4% cholate, 0.5 M NaCl and 1 mM EDTA. The red peak fraction (8 ml) that eluted just before the Triton micelles was saved and concentrated to 2 ml using an Amicon ultrafiltration apparatus with a YM2 membrane. The concentrate was again applied to the same Sephacryl column. The red peak fraction (8 ml) was collected and dialyzed against 5 mM Tris-HCl buffer (pH 8.0) for 17 h. Cytochrome *c*-551 was then precipitated by adding ammonium sulfate to 60% saturation. The precipitate was resuspended in 5 mM Tris buffer and dialyzed again for 10–15 h. PS3 cytochrome *c*-551 can be kept frozen at −80 °C until use.

Results and Discussion

Occurrence of cytochrome c-551

Almost all cytochromes of the Gram-positive thermophilic bacterium PS3, if not all, are membrane-bound. Of these, only one kind of *c*-type cytochrome can be extracted by a mild detergent such as cholate. The cholate-soluble fraction showed an absorption peak at 551 nm as a reduced α band. The amount of this

TABLE I

Effects of aeration on amounts of cytochrome c-551 and cytochrome aa_3 in the membrane fraction

Condition	Amounts in membranes [a] (nmol/mg)	
	c-551	aa_3
Sufficiently aerated	0.15	0.25
Air-limited 1	0.52	0.10
Air-limited 2	1.50	0.02

[a] Obtained from data of the redox difference spectra of the cholate-extractable fraction for cytochrome *c*-551 and the Triton X-100 fraction for cytochrome aa_3.

cytochrome *c*-551 in the cholate extract was very dependent on the extent of aeration. Table I shows the amounts of cytochrome *c*-551 and cytochrome aa_3 in the cells under different aeration conditions. The air-limited cells produced cytochrome *c*-551, while sufficiently aerated cells produced cytochrome aa_3, but very low amount of cytochrome *c*-551.

Purification

The membrane fraction of PS3 cells grown under the air-limited conditions (red membrane), which is rich in *b*- and *c*-type cytochromes [1], was used for the preparation of cytochrome *c*-551. Results of a typical purification of cytochrome *c*-551 from the thermophilic bacterium PS3 are summarized in Table II. Cholate extraction of the red membranes solubilized cytochrome *c*-551 preferentially. A fairly large amount of poly(ethylene glycol) (30%) was necessary to precipitate this cytochrome. The red precipitate solubilized in Tris-HCl buffer (pH 8.0) containing Triton X-100 was fractionated on a DEAE-cellulose column. The affinity of the cytochrome to DEAE-cellulose is not great. Although cytochrome *c*-551 does not look to be effectively purified by these steps (steps 3 and 4), the steps remove the contaminating low-molecular-weight proteins. A gel-filtration column in the presence of 0.4% cholate effectively removed impurities that were found in the excluded and high-molecular-weight fractions. At the final step, cytochrome *c*-551 was precipitated by the addition of ammonium sulfate after most of cholate had been removed. The precipitated cytochrome was solubilized with 5 mM Tris buffer without adding any detergent. However, extensive dialysis against the buffer made the cytochrome solution turbid, but this readily became clear by the addition of cholate or Triton X-100. It is thus likely that the cytochrome itself is hydrophobic, and a small amount of detergent is required to keep the cytochrome in solution.

TABLE II

Purification of PS3 cytochrome c-551

Step	Protein (mg)	Amount of *c*-551 (nmol)	Specific content of *c*-551 (nmol/mg)
Red membrane	2150	–	–
1 Cholate extract	615	1101	1.79
2 PEG fractionated	300	605	1.68
3 1st DEAE-cellulose	95	375	4.0
4 2nd DEAE-cellulose	35	200	5.7
5 Gel filtration	0.54	52	94.1

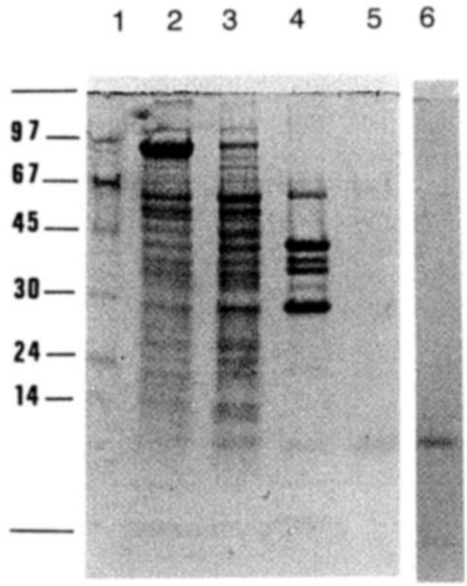

Fig. 1. SDS-gel electrophoresis of PS3 cytochrome *c*-551. The 10% gel [9] was stained with Coomasie blue R-250. The samples are: lane 1, marker proteins composed of phosphorylase *b* (97 kDa); bovine serum albumin (67 kDa), ovalbumin (45 kDa); carbonic anhydrase (30 kDa); trypsin inhibitor (24 kDa) and lysozyme (14 kDa); lane 2, red membranes (50 μg); lane 3, cholate extract (35 μg); lane 4, second DEAE eluate (12 μg); lane 5, the purified sample (1 μg); lane 6, the purified sample (3 μg).

Fig. 1 shows the results of SDS-gel electrophoresis during a typical purification. The final preparation of PS3 cytochrome *c*-551 migrate as single band close to the dye front. The molecular mass was 10.4 kDa as judged by comparison of the mobility with those of standard proteins including lysozyme, horse-heart cytochrome *c* (12.3 kDa) and pancreatic trypsin inhibitor (6.5 kDa). Heme staining with *o*-tolidine showed that this cytochrome is predominant *c*-type cytochrome in the cholate extract (data not shown). The low yield of the purified *c*-551 (Table II) may be attributable to incomplete precipitation of the cytochrome by polyethylene glycol (step 2), the rather strict fractionation at the gel filtration step, and a loss during concentration by the Amicon ultrafiltration membrane (step 5).

Absorption spectra and chromophore

Fig. 2 shows the absorption spectra of the reduced (——) and oxidized (- - - - -) form of PS3 cytochrome *c*-551. Peaks of the reduced form were at 416,

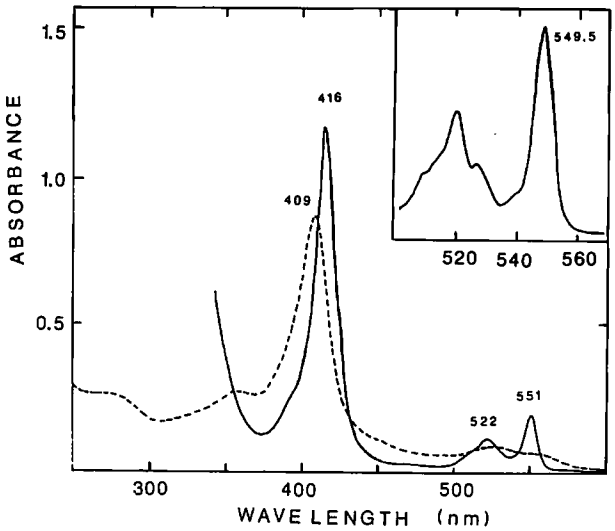

Fig. 2. Absorption spectra of PS3 cytochrome *c*-551. ———, reduced with $Na_2S_2O_4$; - - - - -, oxidized as prepared. Inset: the reduced form at liquid-nitrogen temperature. The sample (86 μg protein/ml) in 30 mM Tris-HCl (pH 8.0) containing 0.1% octaethyleneglycol *n*-dodecyl ether was used.

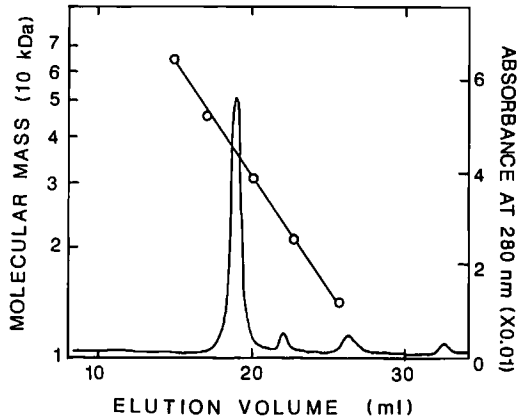

Fig. 3. Determination of molecular mass of PS3 cytochrome *c*-551 in the presence of cholate. A TSK G-3000SW column with 20 mM K·Mops buffer (pH 6.8) containing 0.5 M NaCl, 1 mM EDTA and 0.4% cholate was used. The flow rate was 0.5 ml/min. The standard curve (○) was obtained by using bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme.

522 and 551 nm, while the Soret peak of the oxidized form was at 409 nm. Pyridine ferrohemochrome showed a peak at 550 nm, indicating that the prosthetic group is heme *c*. Quantitative measurement gave molecular absorption coefficients of 20.9 $mM^{-1}cm^{-1}$ for reduced minus oxidized difference at 551–535 nm and 120 $mM^{-1} \cdot cm^{-1}$ at 408 nm for the oxidized form. The absorbance ratio of the reduced form (A_γ/A_α) was 6.1. The heme *c* content was 94 nmol/mg protein, which indicates that one cytochrome *c*-551 molecule contains one molecule of heme *c* and that the preparation of cytochrome *c*-551 was almost pure.

The spectrum of the reduce cytochrome *c*-551 at liquid-nitrogen temperature is shown in Fig. 2, inset. The peak of the α band shifted to 549.5 nm and no extra peak was seen in this region. This characteristic of cytochrome *c*-551 differenciates it from the cytochrome *c*-550 preparation from the thermophilic bacterium PS3, which has been reported to have two peaks, one at 547 and another at 549.5 nm [11].

Some properties

The elution pattern of gel filtration during purification showed that cytochrome *c*-551 moved faster than Triton micelles, indicating that the molecules of the cytochromes may form oligomers. Fig. 3 shows calibration lines for the molecular weight on high-performance gel chromatography with a TSK G3000 SW column and elution pattern of cytochrome *c*-551. The apparent molecular weight of cytochrome *c*-551 was determined to be 33 kDa. These data may indicate either that cytochrome *c*-551 is trimeric in the presence of 0.4% cholate or that dimeric cytochrome *c*-551 binds an appreciable amount of cholate. Similar sized oligomers were also found in the presence of 0.4% lauroyl sarcosinate using the same TSK 3000SW column.

Isoelectric focusing of the partially purified sample (Step 3) using 2% carrier Ampholyte was carried out in the pH range of 3.5–10.0 for 24 h at room temperature. The cytochrome migrated as a single band at an isoelectric point of pH 4.0. Although the affinity of this protein to DEAE-cellulose was not strong, cytochrome *c*-551 is fairly acidic.

One of the important characteristics of a cytochrome is its redox potential (E'_m). The E'_m of cytochrome *c*-551 was measured to be 225 mV. The value is in accordance with the reported values of 221–230 mV for cytochromes *c* in the membrane of PS3 [13].

Role of cytochrome c-551

Recent investigations by this group showed that cells of the thermophilic bacterium PS3 grown under air-limited condition produce cytochrome *o* as one of the terminal oxidases [1,4], whereas sufficiently-aerated PS3 cells use cytochrome aa_3-type oxidase. Cytochrome *c*-551, which is also found in cells grown under air-limited conditions, was oxidized effectively by PS3 cytochrome *o* [4]. Thus it is likely that the respiratory chain of such cells is composed of dehydrogenases, menaquinone, bc_1 complex, cytochrome *c*-551 and cytochrome *o*, since the cytochrome bc_1 complex was also found in cells grown under air-limited conditions. In order to confirm that this alternative respiratory chain exists in air-limited cells, the effect of cholate treatment, which removes cytochrome *c*-551, and the effect of addition of *c*-551 to the depleted membranes were examined. Table III shows that the cholate-treatment lowered the quinol oxidase activity and that the activity was recovered by the addition of purified cytochrome *c*-551. The activity

TABLE III

Effects of addition of cytochrome c-551 to the cholate-extracted membranes

Red membranes (41 mg protein in 1 ml) were extracted twice with 2% cholate in 50 mM Tris-HCl buffer (pH 8.0) and the precipitate isolated after centrifugation of 144000 × *g* for 30 min was resuspended in 1 ml of 10 mM Tris-HCl buffer. The ubiquinol oxidase activity was measured with a 5 μl aliquot of membranes in 3 ml of the reaction medium containing 20 mM sodium phosphate buffer (pH 6.6) as described under Experimental procedures.

Addition	Oxidase activity (nmol/min)
1 Red membranes	26.0
2 Cholate-extracted red membranes	5.2
3 2 + cyt. *c*-551 (1.0 nmol)	10.4
4 2 + cyt. *c*-551 (2.5 nmol)	15.6

remaining after cholate treatment seemed to be due to cytochrome aa_3-type oxidase, since a small amount of cytochrome aa_3 was usually present in such membranes. Fig. 4 shows the effect of cytochrome *c* concentration on the quinol oxidase activity of the mixture of purified cytochrome bc_1 complex and cytochrome *o*. A low concentration of cytochrome *c*-551 was sufficient to reconstitute quinol-oxidase activity. It has already been reported that this cytochrome *c*-551 is readily oxidizable with PS3 cytochrome *o* with a relatively low K_m of 1.3 μM [4], while its K_m for PS3 cytochrome caa_3 oxidase was about 7–16 μM [2,12]. The latter value (16 μM in Ref. 2) seems, however, an overestimation due to the presence of an insufficient amount of ascorbate, which reduces cytochrome *c*.

What is cytochrome c-551?

By analogy to the mitochondrial respiratory chain, two *c*-type cytochromes, cytochromes *c* and c_1, are

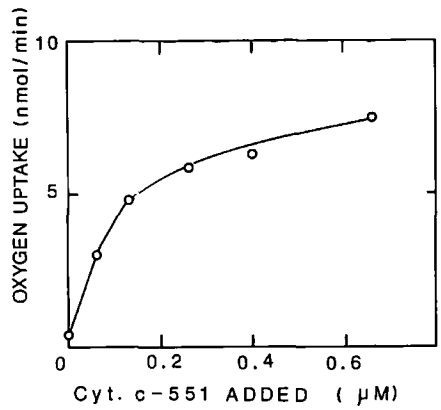

Fig. 4. Mediation of electron transfer between cytochrome bc_1 complex and cytochrome *o* by cytochrome *c*-551. The bc_1 complex (0.27 nmol) supplemented with 1.5 nmol menaquinone-3 and cytochrome *o* (0.22 nmol) was used and the activity was polarographically measured in a medium (3.0 ml) composed of 25 mM K_2SO_4, 2.5 mM $MgSO_4$ and 5 mM sodium phosphate buffer (pH 6.5) at 40 °C.

likely present in Gram-positive bacteria. The thermophilic bacterium PS3 contained cytochrome *c* as a part of subunit II of the aa_3-type oxidase [2,3] and cytochrome $c_1(f)$, as one of the four peptides that constitutes the $bc_1(b_6f)$ complex [6]. The third *c*-type cytochrome is preferentially found in the PS3 cells grown under air-limited conditions. This cytochrome *c*-551 was a small-sized acidic membrane-bound protein extractable with cholate. This cytochrome shows a lower K_m for cytochrome *o* than for cytochrome caa_3, suggesting that cytochrome *c*-551 is the natural substrate of *o*-type oxidase. The presence of cytochrome *c*-551 may be ubiquitous among Gram-positive bacteria, since a variety of Gram-positive bacteria such as *Bacillus subtilis* [14], *Mycobacterium phlei* [15,16] and *Staphyloccus aureus* [17] have been reported to contain cytochrome *o* as well as cytochrome aa_3-type oxidase.

Several 'soluble' *c*-type cytochromes have been purified from Gram-positive bacteria. From *Bacillus subtilis* *c*-550 and *c*-554 have been purified by Miki and Okunuki [18,19], and they may correspond to cytochrome *c*-550 (the natural substrate of aa_3-type oxidase) and cytochrome c_1 of this bacterium, respectively, as discussed by De Vrij et al. [20]. Three 'soluble' *c*-type cytochromes, *c*-551, *c*-552 and *c*-553, have been purified from *Bacillus lichenformis* by treating the cells with lysozyme and trypsin, and successive ion-exchange chromatography [21]. One of these cytochromes may be analogous to PS3 cytochrome *c*-551, if the other two correspond to cytochrome *c* and cytochrome c_1. These 'soluble' *c*-type cytochromes may be derived from their membrane-bound form like cytochrome b_5 as a result of proteinase digestion [22]. Hon-nami et al. [11] also reported cytochrome *c*-550 from thermophilic bacterium PS3. Since this small-sized cytochrome *c* (10 kDa) obtained from the membrane fraction of PS3, was soluble after purification and its isoelectric point was reported to be 5.6, this cytochrome is apparently different from any of the three *c*-type cytochromes that we have found in PS3 cells. It is noteworthy that the cytochrome *c* part of the caa_3-type oxidase is rather basic because of its high content of lysine residues [3]. We have not yet identified this cytochrome *c*-550 in PS3 cells grown under either sufficiently aerated or air-limited conditions. The possibility that this cytochrome *c*-550 is a proteolytic product of cytochrome *c*-551 seems low, because the absorption spectra of the reduced forms at both room and liquid nitrogen temperatures were different. This cytochrome may be a minor component in PS3. Anyway, it seems likely that at least three membrane-bound *c*-type cytochromes are operating in the thermophilic *Bacillus* PS3.

The relationship between the present cytochrome *c*-551 and any of several small-sized cytochromes *c* found in Gram-negative bacteria is unclear at present. It may be possible to elucidate the interrelationship

after determining the amino-acid sequence of PS3 cytochrome *c*-551, which is presently being undertaken in our laboratory.

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