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A NEW CYTOCHROME *b*-LIKE PIGMENT WITH A PEAK AT 567 nm AND A LOW REDOX POTENTIAL IN DENITRIFYING BACTERIA

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SUMMARY

Dithionite reduced difference spectra of extracts of denitrifying pseudomonads revealed small absorption maxima at 567 and 539 nm, suggestive of α and β bands of a new *b* type cytochrome. The new pigment was present in cells grown both aerobically and anaerobically and was located in the particulate fraction of extracts. These extracts also contained, in much higher concentrations, additional pigments resembling cytochromes c_{553} and b_{559}, which were readily reduced by NADH or endogenous substrates, although a small proportion of the b_{559} required dithionite for complete reduction. In contrast, most of the new 567 pigment was not readily reduced by NADH, succinate, or endogenous substrates, and it was most easily visualized with dithionite in the sample cuvette, and either endogenous substrates or NADH in the reference cuvette. Dyes of low redox potential such as benzyl viologen ($E_{m,7} = -359$ mV), phenosafranine ($E_{m,7} = -250$ mV) and reduced janus green ($E_{m,7} = -225$ mV) could substitute for dithionite as reductant for the new 567 pigment. Cresyl violet ($E_{m,7} = -160$ mV) caused partial reduction. However, redox compounds of higher potential such as reduced indigo carmine, ($E_{m,7} = -125$ mV) reduced methylene blue ($E_{m,7} = -11$ mV), ferrooxalate and ascorbate could not replace dithionite as reductant. Most of the cytochrome b_{559} and the c_{553} were reduced by ascorbate. Thus the new 567 pigment appears to have a midpoint potential between -225 and -125 mV, well below most of the cytochrome b_{559}. The new 567-nm pigment was rapidly oxidized by brief but vigorous aeration and was also slowly and partially re-reduced when concentrated extracts were allowed to stand without aeration. A more complete reduction of the 567 pigment was readily obtained by the addition of a mixture of NADH and FAD. The 567 pigment was observed in several denitrifying pseudomonads, *P. fluorescens*, *P. stutzeri* and also in *Micrococcus denitrificans*, but was not detectable in the non-denitrifiers *Escherichia coli* or *Aerobacter aerogenes*.

INTRODUCTION

Bacteria contain a variety of *b* type cytochromes exhibiting α bands between 557 and 565 nm[1,2]. We have noted in a preliminary abstract that denitrifying species of *Pseudomonas* contain a cytochrome with an unusually high α band at 567 nm and a β band near 539 nm[3]. Failure to observe this cytochrome in the past is due to its

low concentration relative to the conventional cytochrome b_{559} and to the use of conventional difference spectra.

In this communication we show how cytochrome 567 can be visualized using special difference spectra, total (dithionite) reduced *vs* substrate or NADH reduced, instead of conventional difference spectra. In addition, the distribution of the cytochrome among bacteria is examined and the new pigment is partially characterized with respect to location, redox potential, substrate reducibility and oxidation by air.

MATERIALS AND METHODS

Denitrifying *Pseudomonas* species were either obtained from the American Type Culture Collection (ATCC) or isolated and characterized locally and coded DMS. They include *Pseudomonas stutzeri* (ATCC 17588), *Pseudomonas fluorescens* (DMS 10), and an isolate termed *Pseudomonas* DMS 2 which resembles *P. denitrificans*. We also examined a denitrifying species from another genus, *Micrococcus denitrificans* (ATCC 13543), and two non-denitrifying bacteria, *Escherichia coli* K-12 and *Aerobacter aerogenes* (DMS 202). Organisms were grown at 32 °C in trypticase soy broth (Baltimore Biological Laboratories). Aerobic growth was obtained using 450 ml of medium in a 2-l Erlenmeyer flask by vigorous aeration, usually for 6 h, in a rotary shaker. The inoculum was 5 ml of a culture grown under the same conditions for 16 h. For anaerobic growth, 2-l Erlenmeyer flasks were completely filled with the same medium supplemented with 0.4% KNO_3 and incubated for 16 h when nitrogen evolution became vigorous.

Cells were harvested by centrifugation, washed in 0.01 M phosphate buffer, pH 7, suspended in twice their volume of 0.05 M phosphate buffer and disrupted by sonification (at 5–10 °C) with five 15-s bursts at 2-min intervals (Sonifier model S-125, Branson Instruments, Inc., Danbury, Conn.) at a power setting of 4 A. Broken cells and larger debris were removed by centrifugation at $23500 \times g$ for 25 min (at 0 °C) and the supernatant fluid was termed the crude extract. Protein was determined by the method of Lowry *et al.*[4].

Difference spectra were recorded with a Cary Model 14 spectrophotometer equipped with a variable intensity light source and a slide wire giving full scale deflection of 0 to 0.1 *A*. 3 ml of extract were placed in cuvettes of 1 cm light path and difference spectra were recorded at room temperature. Thunberg cuvettes, which had been gassed with purified nitrogen, were used when anaerobic conditions were required.

RESULTS

Discovery of cytochrome 567 in cells grown under various conditions

In studying the shifts under various growth conditions in the kinds and amounts of the various cytochromes in denitrifying pseudomonads, we noted that the crude extracts of young (6 h) highly aerobic cells of *Pseudomonas stutzeri* (ATCC 17588) and *Pseudomonas fluorescens* (DMS 11) showed a small peak at 566 nm in conventional difference spectra (Fig. 1). This was surprising because neither we nor others had observed this component in older aerobic cells or in cells grown anaerobically under denitrifying conditions with nitrate as the terminal electron acceptor. To

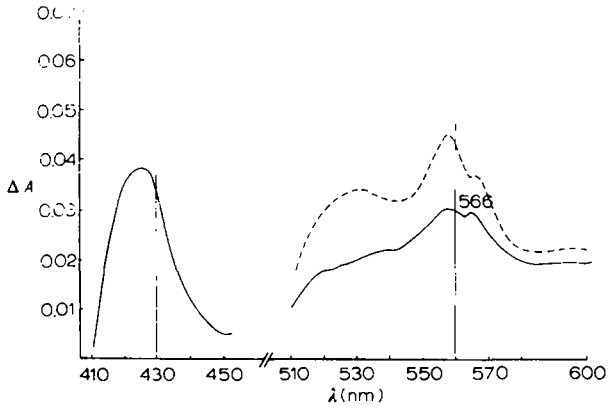

Fig. 1. Difference spectra of crude extracts from young, highly aerobic cells of *P. stutzeri* and *P. fluorescens*. Crude extracts of *P. stutzeri* (25 mg/protein/ml) (– – –) and *P. fluorescens* (20 mg protein/ml) (— ——) were placed in the sample and reference cuvette and dithionite was added to the sample cuvette.

determine whether this cytochrome was actually present under other growth conditions but obscured by large amounts of known *b* and *c*-type cytochromes, we attempted a selective reduction by NADH of the conventional cytochromes in an extract of *P. stutzeri* harvested in the late log phase of aerobic growth (Fig. 2). The selective reduction of conventional *c*- and *b*-type cytochromes revealed a peak at 566 nm and the Soret band split showing two major maxima which could be measured without diluting the extract.

Demonstration of the high wave length cytochrome was more difficult in

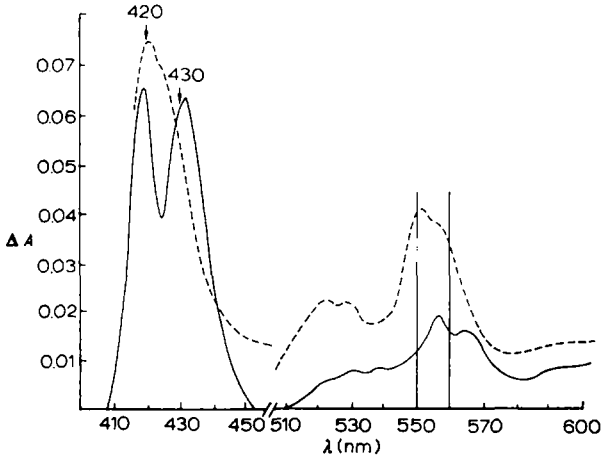

Fig. 2. Effect of NADH on difference spectra of crude extracts of *P. stutzeri*. ———, the reference cuvette was reduced with 1.6 mM NADH and dithionite was added to the sample cuvette. Extract was present at 16.6 mg/ml throughout the entire spectrum. – – –, nothing was added to reference cuvette. Above 500 nm, the extract is present at 8.3 mg protein per ml; below 500 nm, at 0.7 mg per ml.

extracts of *P. stutzeri* grown anaerobically because of the large amounts of cytochrome *c*. Nevertheless it could be observed in the dithionite reduced *versus* NADH reduced type of difference spectrum because the cytochrome *c* was almost totally reduced by NADH.

Cytochrome 567 obtained by differential centrifugation

When crude extracts of *Pseudomonas* DMS 2 were centrifuged for 2.5 h at $100000 \times g$, three fractions were obtained. The clear supernatant fluid contained principally cytochrome *c*, the compact gelatinous pellet at the base of the centrifuge tube exhibited cytochromes *c* and the conventional cytochrome *b*, and the deep-red fraction just above the solid pellet exhibited a conventional cytochrome *b* and the new high wave length 567 *b*-type cytochrome. When this fraction (hereafter referred to as the small particle fraction) was suspended in a minimal amount of 0.05 M phosphate buffer, the new pigment could be visualized spectrophotometrically in conventional difference spectra without adding NADH to the reference cuvette (Fig. 3). The α peak previously observed at 566 nm could be assigned the more accurate wave length, 567 nm, and the β peak showed a maximum at 539 nm. In addition, an asymmetry in the 430–435 nm region of the Soret band became apparent.

Distribution of cytochrome 567 among bacteria

When examined by the techniques indicated in Fig. 3, all of the denitrifying bacteria studied with the possible exception of a strain of *P. aeruginosa* exhibited a characteristic peak at 567 nm. One species, *Micrococcus denitrificans*, had a cytochrome with a slightly different α maximum at 566 nm. It is probably identical with a cytochrome noted at 565 nm by Scholes and Smith[5] in this microorganism. The largest quantity of cytochrome 567 was found in *Pseudomonas* DMS-2 and for this

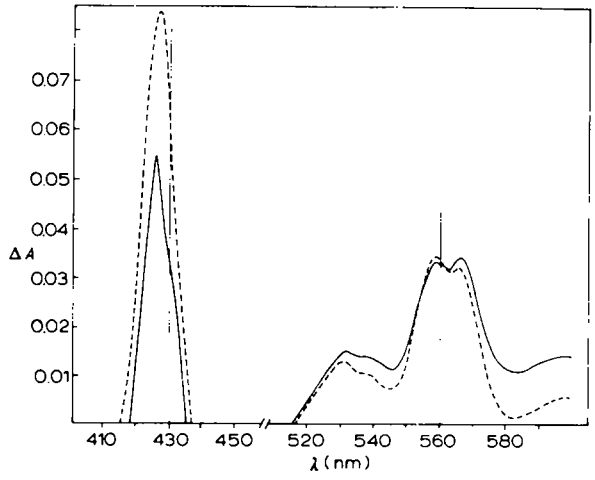

Fig. 3. Difference spectra of the small particle fraction from *Pseudomonas* DMS 2. – – –, sample cuvette reduced with dithionite, reference cuvette with no additions. ——, sample cuvette reduced with dithionite, reference cuvette reduced with 1.6 mM NADH. For spectra above 500 nm, the extracts contained 20.4 mg protein/ml. Below 500 nm, these extracts were diluted to 10.2 mg protein/ml.

reason it served as our chief experimental material. Similarly prepared extracts of the non-denitrifying organisms *E. coli* and a species of *Aerobacter aerogenes* exhibited no peak near 567 nm, although a typical cytochrome *b* peak at 558 nm was evident. Further details on the distribution of the new 567 cytochrome among various species of bacteria will be the subject of a future communication.

Difference spectra of small particle fraction after reduction with dithionite and oxidation with air and ferricyanide

For these experiments, very concentrated small particle fractions from *Pseudomonas* DMS 2 were used. These extracts exhibited a more potent endogenous reducing power than those described above, as indicated by the immediate reduction of added methylene blue and the partial reduction of added benzyl viologen ($E_{m,7} =$

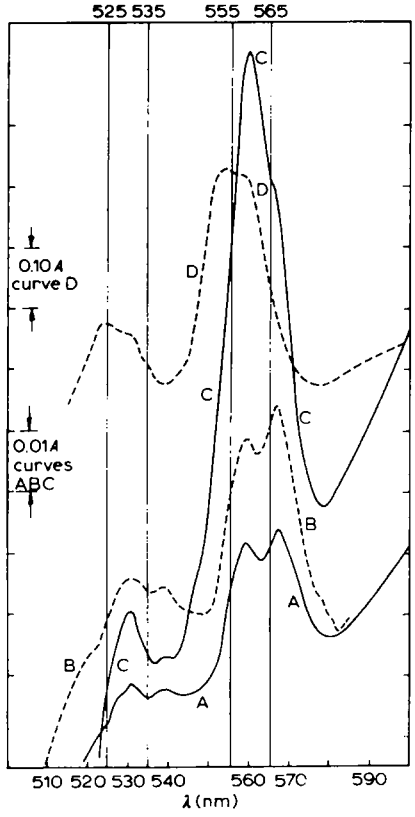

Fig. 4. Oxidation by air and ferricyanide and reduction by dithionite of cytochromes in a very concentrated small particle fraction. Extract (71 mg protein per ml) from *Pseudomonas* DMS 2 was placed in both sample and reference cuvettes and incubated for several minutes at room temperature. Curve A, a few crystals of dithionite were added to the sample cuvette; Curve B, extract in the reference cuvette was vigorously aerated for 1 min, and the spectrum was recorded within 1 min; Curve C: 0.05 ml of saturated solution of potassium ferricyanide was added to reference cuvette; Curve D, additional 0.1 ml of ferricyanide solution was added to reference cuvette; Curve D: additional 0.1 ml of ferricyanide solution was added to reference cuvette. For Curves A, B and C, each absorbance division equals 0.01 *A*; for Curve D, each division equals 0.01 *A*.

−359 mV) four min after their addition to the cuvettes. Difference spectra (Fig. 4) with unaerated concentrated extract in the reference cuvette and dithionite in the sample cuvette (Curve A) revealed the four small but distinct maxima at 567, 559, 539 and 531 nm, indicative of b_{567} and b_{559}. Vigorous aeration or addition of ferricyanide to the reference cuvette (Curves B and C) caused some oxidation of these pigments. Further addition of ferricyanide to the reference cuvette (Curve D, measured on a contracted absorbance scale), revealed a high concentration of cytochrome c_{553} which had been completely reduced by endogenous substrates. The cytochrome b_{559} was also present in high concentration and was mostly reduced by endogenous substrates. However, about 5 to 10% of the 559 pigment required dithionite for reduction. The 567 pigment is obviously present in much lower concentrations. Although complete reduction of this pigment requires addition of dithionite, endogenous substrates in this concentrated extract appear to cause about a 50% reduction.

Oxidation and reduction of the 567 component by aeration and anaerobic incubation of concentrated small particle fraction

With dithionite in the sample cuvette, when the extract in the reference cuvette was vigorously aerated and scanned very rapidly, a large portion of the 559 pigment appeared capable of rapid oxidation by air and rapid initial reduction by endogenous substrates (Fig. 5). The 567 pigment was also rapidly oxidized by aeration, but an initial rapid reduction by endogenous substrates could not be demonstrated, although partial reduction (less than 50%) may be masked by the changes at 559 nm. Slow but incomplete reduction of the 567 pigment and of an additional quantity of the 559 cytochrome occurs over a 60-min period (Fig. 5).

Attempts to establish the redox potential of the 567 component by reduction with compounds of various redox potential

Although an exact determination of redox potential has not been possible in these concentrated small particle preparations, certain dyes of sufficiently low redox potential can substitute for dithionite in causing reduction of the 567 nm component. In these experiments, the redox indicators were added to the sample cuvette under anaerobic conditions, and the reference cuvette contained extract with no additions. The amount of cytochrome reduction caused by each indicator was compared to complete reduction caused by dithionite. Neither ascorbate (5 mM) nor potassium ferrooxalate (1 mM) could replace dithionite as reductant, suggesting a lower redox potential than is exhibited by most conventional cytochromes of the *b* type. However, benzyl viologen (17 μg/ml) ($E_{m,7} = -359$ mV), phenosafranine (1 nM) ($E_{m,7} = -252$ mV)[6] and janus green B (2.7 nM) ($E_{m,7} = -225$ mV)[6] caused apparently complete reduction of the 567 component, although these dyes were themselves only partially reduced. The extent of dye reduction by endogenous substrates was estimated by measuring the decrease in absorbance at the absorption maximum of the dye. Methylene blue (215 nM) ($E_{m,7} = -11$ mV) and indigo carmine (5 nM) ($E_{m,7} = -125$ mV)[6] were completely unable to replace dithionite as reductant for b_{567}, even after 90 min of incubation, although the dyes themselves were completely reduced. Cresyl violet (20 nM) ($E_{m,7} = -160$ mV)[7] was partially able to replace dithionite, causing between 60 and 80% of cytochrome b_{567} reduction, although the dye itself was more than 95% reduced. Although further studies are needed, these

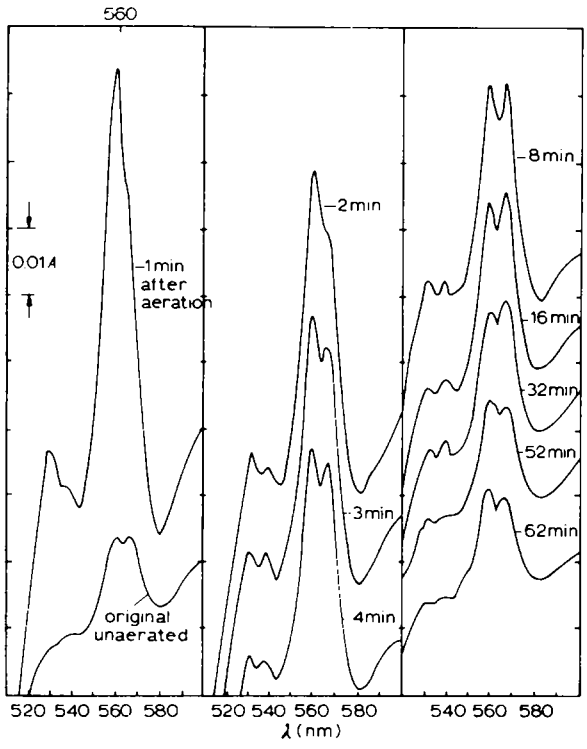

Fig. 5. Effect of aeration and anaerobiosis on the 567- and 559-nm pigments. The concentrated small particle fraction (62.5 mg protein/ml) was placed in both the sample and the reference cuvette. After standing in the covered cuvette for 2 h at room temperature, dithionite was added to the sample cuvette and the spectrum was recorded (original, unaerated). Then the contents of the reference cuvette was transferred rapidly to an Erlenmeyer flask, shaken in air for 5 s, and returned immediately to the reference cuvette. The spectrum was again rapidly recorded at various time intervals beginning 1 min after aeration, and continuing at the time intervals shown. After 62 min, the original spectrum is essentially restored.

data are most compatible with a midpoint potential for cytochrome b_{567} between that of phenosafranine (−225 mV) and indigo carmine (−125 mV).

It was interesting to find that in addition to reduction by these low potential artificial dyes, the 567 component could also be reduced if both FAD (0.1 mM) and NADH (4 mM) were added to the sample cuvette (Fig. 6). Although ascorbate could not replace dithionite as reductant for the 567 component, it did reduce at least 90% of cytochrome c_{553} and about 80% of the total amount of pigment absorbing at 559 nm. This was determined using a different and more dilute large particle fraction, which contained little endogenous reducing power.

DISCUSSION

The peaks in difference spectra at 567 and 539 nm appearing upon reduction with dithionite suggest α and β bands of a cytochrome pigment, probably one of the *b* type, on the basis of wavelength[2] and midpoint potential lower than found for

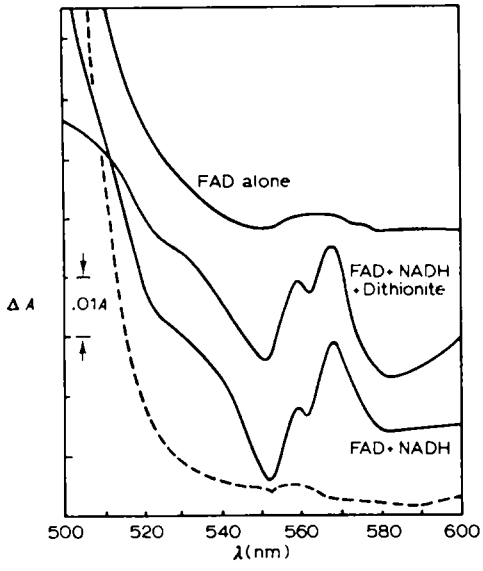

Fig. 6. Reduction of 567 pigment by FAD *plus* NADH. The concentrated small particle fraction (35 mg protein/ml) was placed in both sample and reference cuvettes. Where indicated, NADH (2 mM), FAD (0.1 mM), or dithionite were added to the sample cuvette and the spectrum was scanned. Spectra illustrated by ——— all contain extract in both cuvettes. – – –, the spectrum of this concentration of FAD in buffer (0.05 M phosphate, pH 7.0) measured against a buffer blank.

cytochromes of the *c* or *a* type. The Soret peak of the 567 pigment is probably between 430 and 435 nm.

While the apparent low redox potential (between −125 and −225 mV) of the 567 component may hamper its complete reduction by NADH, succinate or endogenous substrates, our inability to demonstrate substrate reduction raises the question of the function of this pigment in the respiratory system of the cell. The observation that NADH, when added together with high concentrations of FAD, can cause reduction of the 567 pigment, might indicate that the pigment is in equilibrium with unidentified low potential flavins or flavoproteins *in vivo*. Perhaps this cytochrome *b* may serve as a low potential carrier in the process of denitrification in denitrifying bacteria. Ferredoxin and a low potential cytochrome *c* (ref. 8) are other low potential carriers found in other bacteria.

In difference spectra, the appearance of the 567 component is always accompanied by a peak of about equal height at 559 nm. It seems most likely that this peak consists of at least two separate *b* cytochromes, with the major component resembling a conventional cytochrome *b* of relatively high midpoint potential, and the minor component sharing many of the properties of the 567 component including lack of substrate reducibility and low midpoint potential.

It is timely to find a *b*-type cytochrome in denitrifying bacteria which exhibits properties of high wavelength α band and low redox potential. There is much current interest in a cytochrome *b* from mammalian and yeast mitochondria exhibiting a high α band (565 or 566 nm) and a comparatively low midpoint potential (−30 to −50 mV)[9–12]. A cytochrome *b* pigment in the photosynthetic bacteria *Rhodospirillum*

rubrum and *Rhodopseudomonas spheroides* also exhibits a midpoint potential more negative than −90 mV and α peaks at 562 to 565 nm[7,13,14].

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