Immunological Investigation of the Distribution of Cytochromes Related to the Two Terminal Oxidases of Escherichia coli in Other Gram-Negative Bacteria

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Monospecific antibodies were raised against the two terminal oxidase complexes of the aerobic respiratory chain of Escherichia coli. These are the cytochrome d and cytochrome o complexes. The antibodies were used to check for the occurrence of cross-reactive antigens in membrane preparations from a variety of gram-negative bacteria by rocket immunoelectrophoresis and immunoblotting techniques. With these criteria, proteins closely related to the cytochrome d complex of E. coli appeared to be widely distributed. Among the strains containing cytochrome d-related material were Serratia marcescens, Photobacterium phosphoreum, Salmonella typhimurium, Klebsiella pneumoniae, and Azotobacter vinelandii. The data suggest that the d-type terminal oxidase in many of these strains is associated in a complex with b-type and a-type cytochromes, as has been found to be the case in E. coli. K. pneumoniae and S. typhimurium were also shown to have material cross-reactive to the E. coli cytochrome o complex.

The aerobic respiratory chain of Escherichia coli is branched and contains two terminal oxidases, the cytochrome d complex and the cytochrome o complex (10). Both of these oxidases have been purified to homogeneity (25, 26, 31, 33, 37) and have been shown to carry out electrogenic reactions in reconstituted proteoliposomes (24, 28, 33). Electron flow through either oxidase generates a transmembrane voltage difference. Whereas the cytochrome aa3-type terminal oxidases oxidize ferrocyanochrome c, the oxidases of E. coli appear to directly oxidize ubiquinol in the bacterial membrane (25, 28). The cytochrome o complex predominates in the E. coli membrane when cells are grown with high aeration (31), whereas the cytochrome d complex, which has a higher affinity for oxygen (44), is induced when cells are grown under oxygen-limiting conditions (31; see also references 10, 20, and 41).

The cytochrome d complex has been shown to contain three cytochrome components, b558, a1, and d, but only two polypeptides by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (26, 27, 37). The larger of the two subunits (subunit I; molecular weight, 57,000) has been shown to contain the cytochrome b558 component of the complex (8). One question of interest which is addressed in this work is whether other gram-negative bacteria, many of which contain d-type cytochrome by spectroscopic criteria (20, 41), contain immunocross-reactive material to either or both of these subunits. This was determined by using antibodies raised specifically against either subunit I or subunit II (molecular weight, 45,000) of the cytochrome d complex of E. coli.

The cytochrome o complex of E. coli contains heme b (25, 31, 33) and copper (25) and is reported to have two (25) or four (31, 33) subunits by SDS-PAGE analysis. Antibodies raised against the native oxidase were used to check for cross-reactivity with preparations from various gram-negative bacteria. Several of these strains contain o-type terminal oxidases by spectroscopic criteria, and it was interesting to investigate their immunological relationships.

MATERIALS AND METHODS

Organisms and growth conditions. The bacterial strains used in this work are listed in Table 1. The isolates were taken from stocks used for teaching purposes, and most were originally obtained from the American Type Culture Collection. Before use, the strains were checked by metabolic characterization tests. Most bacterial strains were grown in Penassay broth (50 ml or 100 ml; Difco Laboratories, Detroit, Mich.) in 250 ml Klett flasks. The flasks were shaken at 200 rpm at the recommended temperature for each strain, and the cells were harvested at stationary phase. Photobacterium phosphoreum required 3% NaCl in the medium. Several strains, Rhodopseudomonas palustris, Rhodospirillum rubrum, Rhodospirillum fulvum, and Rhodopseudomonas sphaeroides, were grown in Sistrom basal medium (47). Rhodospirillum fulvum was grown microaerophilically in the dark for 2.5 days in medium supplemented with 5 μg of para-aminobenzoic acid per ml. Pseudomonas putida (48), Klebsiella aerogenes (11), and Paracoccus denitrificans (12) were grown in media and conditions previously described. Azotobacter vinelandii (19) was grown microaerophilically in Winogradsky medium with sucrose, agar, and molybdenum. E. coli SHSP19 (45), which does not synthesize heme, was grown semiaerobically.

Immunological methods. The antibody preparations used in this work have all been previously described. Polyclonal antibodies against E. coli cytochrome o were raised against immunopurified cytochrome (31). Polyclonal antibodies were raised against subunit II of the cytochrome d complex, which had been excised from an SDS-polyacrylamide gel after electrophoresis (29). A monoclonal antibody (A14-5) which has been shown to react with subunit I of the cytochrome d complex was also used (32).

Harvested cells were washed and disrupted by sonication. Membranes were prepared and solubilized by using Zwittergent 3-12 as previously described (31). For SDS-PAGE immunoblotting (2), samples containing 60 μg of membrane...
protein each were used. Details are given elsewhere (29). For dot immunoblotting, samples of Zwittergent 3-12 solubi-
lized membranes (50 μg of protein) were first mixed with
SDS-PAGE sample buffer and then filtered through nitrocel-
lulose labeled with 125I-protein A and autoradiographed as
described previously (30). Rocket immunoelectrophoresis
was performed as before (31) with the Zwittergent-solubi-
lized membranes (approximately 100 μg of protein).

RESULTS AND DISCUSSION

Cytochrome d complex. A number of bacteria other than E.
coli have been reported to contain cytochrome d (previously
called cytochrome a2) based on spectroscopic criteria (20,
41). In addition to the strains listed in Table 1, other strains
have also been reported to have cytochrome d, including
Haemophilus parainfluenzae (15), Achromobacter strain D
(1), and Pasteurella tularensis (6). In most cases the pres-
ence of cytochrome a1 in the membrane correlates with the
presence of cytochrome d (20, 41). Very few studies have
included biochemical characterization, although a study has
been reported on the cytochrome d from Photobacterium
phosphoreum (54). Often the amount of cytochrome d can be
optimized by using selective growth conditions, usually
limited-oxygen conditions. No attempt was made in the
current work to optimize for cytochrome d production in all
the bacterial strains which were examined. Figure 1 shows
an SDS-PAGE immunoblot of membrane preparations with
monoclonal antibodies directed against subunit I of the
cytochrome d complex (32). Many of these strains contained
a protein closely related to subunit I of the E. coli cyto-
chrome d complex, which has previously been shown to be
cytochrome b558 (8). Most strains with cytochrome d by

![FIG. 1. SDS-PAGE immunoblotting of membrane preparations from various gram-negative bacteria with a monoclonal antibody preparation directed against subunit I of the cytochrome d terminal oxidase complex of E. coli K-12. Lanes: 1, E. coli K-12 (MR43L); 2, Serratia marcescens; 3, Enterobacter aerogenes; 4, Pseudomonas putida; 5, Proteus vulgaris; 6, Pseudomonas fluorescens; 7, E. coli W191-6; 8, Proteus mirabilis; 9, Pseudomonas aeruginosa; 10, Rhodopseudomonas palustris; 11, Paracoccus denitrificans; 12, Photobacterium phosphoreum; 13, E. coli HSHP19; 14, Rhodospirillum rubrum; 15, Rhodospirillum fulvum; 16, Rhodopseudomonas sphaeroides; 17, Arthrobacter pyridinolis; 18, Acinetobacter HOIN; 19, Klebsiella pneumoniae; 20, Salmonella typhimurium; 21, Azotobacter vinelandii; 22, Vitreoscilla sp.](http://jb.asm.org/Downloaded from http://jb.asm.org/on November 6, 2017 by guest)
spectroscopic criteria contained this subunit. It is reasonable to conclude that a b-type cytochrome is associated with cytochrome d in the other bacterial strains as it is in E. coli. The presence of the bands in Fig. 1 other than that identified as subunit I does not reflect a lack of specificity of the antibody, which was a monoclonal antibody. Bands below subunit I (e.g., lanes 1 and 3) are due to proteolysis and have been previously noted (32). Occasional smearing above subunit I (e.g., lanes 19 and 20) probably resulted from a tendency of this protein to aggregate, which has also been previously observed (37). Note that the sample from Arthrobacter pyridinolis (Fig. 1, lane 17) ran anomalously, with most of the protein migrating as a single band. Blotting results were probably due to nonspecific trapping in this case. In all other cases, the membrane proteins were well resolved on the gel, as indicated by protein staining with Coomassie blue, and the blotting results were quite specific. The five strains reported to contain no cytochrome d by spectroscopic criteria (Table 1) also contained no cross-reactive material (Fig. 1).

Immunoblotting after SDS-PAGE with anti-subunit II of the cytochrome d complex showed clear evidence of subunit II in close relatives of E. coli, including Enterobacter aerogenes, Klebsiella pneumoniae, and Salmonella typhimurium. However, subunit II does not transfer efficiently from the polyacrylamide gel to the nitrocellulose, and this could result in false negative results. In order to avoid this step, dot immunoblotting was used. Fig. 2 shows that all these strains which had a protein which cross-reacted to subunit I also had material cross-reactive to subunit II. A. pyridinolis did not appear to contain either subunit, at least not when grown under the conditions we used in these experiments. Vitreoscilla sp. was not reported to contain cytochrome d, and this is consistent with the data in Fig. 2; thus, Vitreoscilla sp. served as a negative control.

Immunoprecipitation studies were also performed with antibodies against the native cytochrome d complex from E. coli. Only in the case of E. coli was 100% of the cytochrome d precipitated by this antibody (not shown). In most other cases, very little cytochrome d was precipitated from a Zwittergent 3-12 solution after the addition of the antibody preparation. These findings suggest significant divergence between the E. coli enzyme and the cytochrome d present in the other strains.

Another experiment was performed to examine the membranes of E. coli strain SHSP19 (45). This strain does not synthesize heme in the absence of 8-amino levulinic acid. It has been reported that membranes prepared from this strain must contain cytochrome apoproteins because respiratory oxidase activity can be reconstituted with the addition of hematin and ATP (43). The immunoblotting results (Fig. 1) showed that the cytochrome d complex was present in the membrane, even though no heme was present.

Cytochrome o complex. Immunocross-reactivity data with cytochrome o are less complete than those obtained with cytochrome d because the antibody preparation against cytochrome o did not immunoblot well. Experiments were thus limited to rocket immunoelectrophoresis, which requires immunoprecipitation. As was seen with the anticytochrome d, it is likely that some strains may contain cross-reactive material which would be manifest in immunoblotting experiments but which will not be apparent by immunoprecipitation. The data (Fig. 3) show that cytochrome o similar to that found in E. coli is in close relatives, including E. aerogenes, K. pneumoniae, and S. typhimurium. In each of these cases the major immunoprecipitin arc stained for the presence of heme, confirming that the cross-reactive component is a cytochrome. The minor arcs which were observed in some cases did not contain heme. All other strains failed to show any heme-staining rocket immunoprecipitin arc, suggesting that there were no proteins strongly cross-reactive to cytochrome o. This would indicate that the
cytochrome o species reported in many of these organisms are not very closely related to the E. coli enzyme.

Note that most of the cytochrome o species which have been purified from organisms other than E. coli either function as cytochrome c oxidases or contain a c-type cytochrome as an apolightly bound component. These include cytochrome o from Rhodopseudomonas sphaeroides, (50, 51), R. capsulata (16, 17), R. palustris (23), P. aeruginosa (29, 55), A. vinelandii (56, 57, 58), and Methylphilus methylophilus (3). Hence, these may be quite distinct from the E. coli enzyme which neither contains a c-type cytochrome nor has cytochrome c oxidase activity (12, 29, 33). The well-characterized cytochrome o of Vitevescia sp. is a soluble enzyme of unknown function (52, 53) and is probably not closely related to the E. coli enzyme. E. coli SHSP19 (heme deficient) was also examined by rocket immunoelectrophoresis for the presence of apo-cytochrome o. Surprisingly, no rocket was apparent, suggesting the absence of cytochrome o when the strain is not synthesizing heme. Others (43) have presented electrochemical evidence that apocytochrome o must be present in membranes of cells unable to synthesize heme. Further studies will be required to clarify this situation. Possibly deterrent solubilization results in denaturation of the cytochrome in the absence of heme.

In summary, the data presented here clearly show that some of the close relatives of E. coli contain both of the terminal oxidases characterized in E. coli and that at least the cytochrome d complex is widely distributed among gram-negative bacteria. Furthermore, subunits I and II of the complex appear always to be present together, suggesting an association of the b-type cytochrome (i.e., subunit I) with cytochrome d in all the cases examined. Presumably, cytochrome a1 is associated in a complex with cytochrome d in these other bacterial species as well. In many cases the published reduced-minus-oxidized difference spectra of membrane preparations from these bacteria show cytochrome a1 along with cytochrome d (20, 41).

Finally, the immunoblotting data show that the apo-cytochrome d complex is synthesized and inserted into the membrane in the absence of heme biosynthesis. These findings complement the work of others and show that other apocytochromes are present in the membranes of heme-deficient E. coli (43).

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LITERATURE CITED

Escherichia coli aerobic respiratory chain. II. Purification and properties of cytochrome b_{553}d complex from cells grown with limited oxygen and evidence of branched electron-carrying systems. J. Biol. Chem. 259:3375–3381.


