Characterization of YpmQ, an Accessory Protein Required for the Expression of Cytochrome c Oxidase in *Bacillus subtilis*\(^*\)

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A search of the *Bacillus subtilis* genome identifies a potential homolog, *ypmQ*, of the inner mitochondrial membrane protein Sco1 from yeast. Sco1 has been found to aid the delivery of copper to cytochrome *c* oxidase. *B. subtilis* expresses two members of the cytochrome oxidase family, a cytochrome *c* oxidase that has two copper centers, Cu\(_A\) and Cu\(_B\), and a menaquinol oxidase that has only Cu\(_{Bp}\). Deletion of *ypmQ* in *B. subtilis* depresses expression of cytochrome *c* oxidase but not menaquinol oxidase. Levels of cytochrome *c* oxidase recover when copper is added to the growth medium of the Δ*ypmQ* strain or when *ypmQ* is expressed from a plasmid. Neither treatment affects the amount or activity of menaquinol oxidase. YpmQ in which two conserved cysteines are replaced by serines and a conserved histidine is replaced by alanine do not complement the deletion of *ypmQ* even though these mutant forms are found in the membrane extract at a level similar to the wild type protein. We propose that the two cysteines and the histidine are critical for the function of YpmQ and suggest they are involved in copper exchange between YpmQ and the Cu\(_A\) site of cytochrome *c* oxidase.

Cytochrome oxidases are integral membrane protein complexes that catalyze the reduction of oxygen to water and capture some of the redox free energy of this reaction as atransmembrane electrochemical gradient. The key structural features that are shared among all members of this family are known in its largest subunit, subunit I. Subunit I is an integral membrane protein with 12–14 membrane-spanning helical segments that provide binding sites for two heme A moieties, known as cytochrome *a* and cytochrome *a*\(_3\), and one copper center, Cu\(_B\). Cytochrome *a*\(_3\) sits in close proximity to Cu\(_B\) and together they form a dinuclear site that is responsible for binding oxygen and its partially reduced states, which arise transiently in the course of catalysis. Cytochrome *a* is a low spin heme that functions to deliver electrons to cytochrome *a*\(_3\)-Cu\(_B\) (1).

The nature of the electron input site reflects a division in the cytochrome oxidase family of enzymes into two groups. In cytochrome *c* oxidases, such as the enzyme found in the mitochondrial inner membrane of eukaryotes, reducing equivalents are delivered from the soluble protein ferrocytochrome *c*. The cytochrome *c* interaction site on the oxidase is predominantly defined by subunit II (2, 3). Subunit II has two transmembrane helices that anchor a solvent-exposed domain, which provides the inner sphere ligands for the dinuclear Cu\(_A\) center. Although there is no direct structural information on a cytochrome *c*-cytochrome *c* oxidase complex, kinetic (4, 5), mutagenic (6, 7), and modeling (8) studies indicate that cytochrome *c* binds at a site near the Cu\(_A\) center to allow for efficient electron transfer from cytochrome *c* to Cu\(_A\). Thus, electrons enter cytochrome *c* oxidase via Cu\(_A\) and are transferred to the cytochrome a\(_5\)-Cu\(_B\) center through cytochrome *a* (9). The second group within the cytochrome oxidase family is the quinol oxidases, which receive reducing equivalents from a lipid-soluble quinol. The best known of this group is the ubiquinol oxidase from *Escherichia coli* (10). Even though the quinol oxidases do not oxidize cytochrome *c* they do have a subunit II that has overall homology with the subunit II of the cytochrome *c* oxidases. The major difference in subunit II of the quinol oxidase is the lack of the amino acid ligands for the Cu\(_A\) center. The lack of Cu\(_A\) accounts for the lack of reactivity of the quinol oxidases with cytochrome *c*.

Copper is an element that is used in proteins to fulfill specific catalytic and structural roles. However, copper is also a potential danger in biological systems due to its ability to catalyze oxidative damage of many cellular components. A new class of proteins, metallochaperones, have been identified that mediate the incorporation of copper into a variety of specific binding sites (11). As outlined above, the integral membrane enzyme cytochrome *c* oxidase has two biochemically and physically distinct copper centers. The Cu\(_A\) center is composed of two copper ions that are held by a set of amino acid ligands such that the coppers are within bonding distance from one another (12). The second copper center of cytochrome *c* oxidases is known as Cu\(_B\) and is physically associated with cytochrome *a*\(_3\). Cu\(_A\) is contained in the extra-membranous domain of subunit II, whereas Cu\(_B\) is found in the membrane-embedded domain of subunit I (13, 14). There is much known about the role of these copper centers in the catalytic cycle of the enzyme, but relatively little is known about the mechanism of their assembly. A number of protein factors have been proposed to have a role in the assembly of complex integral membrane proteins such as cytochrome *c* oxidase (15). In the last few years some of these assembly factors have been more specifically defined. A pathway for the import of copper into mitochondria and its assembly into the copper centers of cytochrome *c* oxidase has been proposed from studies in yeast (16). A pair of copper transporters, CTR1 (17) and CTR3 (18), have been found in the plasma membrane that are responsible for the specific, high affinity uptake of copper. Copper is then passed to a set of soluble binding proteins, one of which is Cox17. Cox17 binds copper in the cytoplasm and moves to the mitochondrial intermembrane space by an as yet unknown mechanism. Another protein Sco1, which is an integral component of the inner mitochondrial membrane, has been implicated in copper delivery to cytochrome *c* oxidase because yeast strains made defi-
having a cytochrome c places it among the cytochrome oxidases. However, subunit II of respiratory chain of the aerobic bacterium Bacillus subtilis does not have a Cu A center, but subunit I of cytochrome oxidase, which is homologous (62% similar) to subunit II of cytochrome oxidase of Bacillus subtilis, has both Cu A and Cu B centers involved in copper center assembly.

Whether Cox17 passes copper to cytochrome c oxidase is unaffected in the same strains. We show that the absence of ypmQ from the genome can be overcome by supplementing the growth medium with copper or by expressing ypmQ from a plasmid. We have made site-directed changes to two conserved cysteine residues and one histidine within the sequence of YpmQ that support a role for these residues as potential copper-binding ligands. It is proposed that YpmQ is involved in the assembly of the CuA center but is not involved in the assembly of the CuB center.

**Experimental Procedures**

**Bacterial Strains and Cultivation Conditions—**B. subtilis and Escherichia coli strains used are listed in Table I. All B. subtilis strains were grown on LB medium at 37 °C; LB agar also contained 1.5% agar (Difco). All B. subtilis strains were grown on super rich medium (22) or supplemented with antibiotic concentrations for phenotype testing and routine growth were as follows: 100 μg/ml ampicillin, 5 μg/ml chloramphenicol, 10 μg/ml kanamycin, 10 μg/ml tetracycline, 7 μg/ml Nm, 100 μg/ml spectinomycin, and 75 μg/ml streptomycin. Antibiotics and other chemicals were from Sigma unless otherwise noted.

**Cloning of ypmQ—**The ypmQ gene was amplified by PCR from B. subtilis 168 genomic DNA template by using primers 1 and 2 (Table II), and inserted into pBluescript KS+ for sequencing. The resulting plasmids were then transformed into DH5α (Str) or E. coli DH5α (lacZ) and grown on tryptic blood agar base (Difco) containing 100 μg/ml ampicillin, 5 μg/ml chloramphenicol, and 10 μg/ml kanamycin. The resulting transformants were confirmed by sequencing. The amplified plasmids were then transformed into B. subtilis strains (Table II) to identify a homolog of Cox17. Here, we show that disruption of the expression of ypmQ results in cytochrome c oxidase-deficient B. subtilis. In contrast, the expression of menaquinol oxidase is unaffected in the same strains. We show that the absence of ypmQ from the genome can be overcome by supplementing the growth medium with copper or by expressing ypmQ from a plasmid. We have made site-directed changes to two conserved cysteine residues and one histidine within the sequence of YpmQ that support a role for these residues as potential copper-binding ligands. It is proposed that YpmQ is involved in the assembly of the CuA center but is not involved in the assembly of the CuB center.

A search of the B. subtilis chromosome reveals a potential homolog of the yeast mitochondrial protein Sco1 but does not identify a homolog of Cox17. Here, we show that disruption of the expression of ypmQ results in cytochrome c oxidase-deficient B. subtilis. In contrast, the expression of menaquinol oxidase is unaffected in the same strains. We show that the absence of ypmQ from the genome can be overcome by supplementing the growth medium with copper or by expressing ypmQ from a plasmid. We have made site-directed changes to two conserved cysteine residues and one histidine within the sequence of YpmQ that support a role for these residues as potential copper-binding ligands. It is proposed that YpmQ is involved in the assembly of the CuA center but is not involved in the assembly of the CuB center.

**Table I**

Plasmids and strains

| Plasmids | Relevant genotype | Ref./Source |
|----------|------------------|-------------|
| pBR322   | Ap, Tc           | 23          |
| pDia5340 | pBR322, goxABCD, Ap | P. Glaser |
| pHP13    | Cm               | 24          |
| pIC156   | Sp cassette      | 30          |
| pNM34    | pBR322, ΔhomHII-SalI, Ap | This study |
| pNM35    | pNM34, goxABCD   | This study  |
| pNM42    | pNM35, 3ox::Sp   | This study  |
| pNM46    | Nm cassette      | This laboratory |
| pNM57    | pBR322, ypmQ, Ap | This study  |
| pNM137   | pHP13, ypmQ      | This study  |
| pNM171   | pHP13, ypmQ      | This study  |
| pNM175   | pNM171, ypmQ, six-histidine tag | This study |
| pNM207   | pNM175, ypmQ, C64S/C68S | This study |
| pNM209   | pNM57, ΔypmQ::Nm | This study  |
| pNM305   | pNM175, ypmQ, H154A | This study |
| pNM315   | pNM175, ypmQ, C68S | This study |
| pNM324   | pNM175, ypmQ, C64S | This study |
| **B. subtilis strains** | | |
| 168      | trpC2            | BGSC |
| BH101    | 168, 3ox::Sp     | This study |
| BH144    | 168, ypmQ::Nm    | This study |
| BH148    | BH144, pNM207    | This study |
| BH149    | BH144, pNM171    | This study |
| BH150    | BH144, pNM175    | This study |
| BH170    | 168, ctaC::Tc    | 49          |
| BH180    | BH144, pNM305    | This study |
| BH181    | BH144, pNM315    | This study |
| BH182    | BH149, ctaC::Tc  | This study |
| BH193    | BH144, pNM324    | This study |
| **E. coli strains** | | |
| DH5α     | endA1 hisdR17 supE44 thi-1 recA1 gyrA relA1 Δ(lacZYA-argF) U169 deoR (Δ80 lacΔ(lacZ) A15) | Life Technologies, Inc. |
| GM2163   | ara-14 leuB6 thi-1 fhuA31 lacY1 tex-78 galK2 galT22 supE44 hisG4 rpsL136 (Sm) y-i5-ntf-1 dam13::Tn9Sm dcm-6 mcrB1 hisdR2 mcrA | 29          |
| TP611    | thi-1 thr-1 leuB6 lacY1 tonA21 supE44 hsdR hsdM recBC top1 lig+ cya-c610 penB | 27          |
TABLE II

| Primer number | Sequence |
|---------------|----------|
| 1             | 5’TAGAATTCATAGCCTGGAGATCATGAGCCTCC3’ |
| 2             | 5’TAGGACACCCAGTATCTTGTGATAGATCCT3’ |
| 3             | 5’CCGATCCTGAATAGCTGACTTGTACGACTGTCCT3’ |
| 4             | 5’AGGCGATTCCTGAGTGACTGCTGCCGCT3’ |
| 5             | 5’TCTTATACGCCGACTGACCC3’ |
| 6             | 5’TTCGAGATGTTGAGATATACATATC’ |
| 7             | 5’TCAATATGTCGCCAATGACC3’ |
| 8             | 5’TCTACACATGTTGAGATATACATATC’ |
| 9             | 5’TCTTATACGCCGACTGACCC3’ |
| 10            | 5’AGCATATATGTCGCCAATGACC3’ |

Nucleotide bases that are in boldface have either been added to or mutated from the wild type ypQ sequence.

YpmQ, an Assembly Factor for Cytochrome c Oxidase

A Blast (33) search of the protein data base derived from the B. subtilis genome (25), using the sequence of the cytochrome c oxidase assembly protein Sco1 from yeast, identifies a possible homolog, YpmQ (Fig. 1, A and B). The same approach does not identify a homolog of the yeast copper chaperone Cox17 in the B. subtilis genome. Analysis of the ypmQ gene finds two potential promoters, ρ0H and ρ1H (34). The ρ1H promoter is probably the most efficient as it has perfect consensus for promoters of this type. Also, ρ1H is produced late in growth, which correlates well with the appearance of cytochrome c oxidase (35). YpmQ is predicted to have a molecular mass of approximately 21.5 kDa and, from hydrophy analysis (36), to contain a single aminoterminal transmembrane-spanning helix, suggesting that it is a membrane-bound protein. Further examination of the predicted transmembrane helix shows that it also contains the sequence of amino acids that specify the covalent attachment of Wurster’s blue radical that results from TMPD oxidation by cytochrome c oxidase (35). YpmQ is an Assembly Factor for Cytochrome c Oxidase

The ypQQ gene was cloned from the chromosome of B. subtilis using PCR to yield pNM87. A Nm antibiotic resistance cassette was used to replace most of the ypQQ gene in pNM87 to transform B. subtilis. Transformation of B. subtilis with pMN209 restores homologous recombination with ypQ in the chromosome to rescue the Nm cassette. Strain BH144 is Nm-resistant and ypQ. A plate assay based on the production of the intensely colored Wurster’s blue radial that results from TMPD oxidation by cytochrome c oxidase was used to test B. subtilis colonies for the presence of cytochrome c oxidase activity (31). It is impor-


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**Fig. 1. Sequence of ypmQ and its relationship to the yeast copper assembly protein Sco1.** A, the ypmQ gene is 579 bp and produces a gene product of 193 amino acids. For reference, the 3′-end of the upstream gene ypmP produces a gene product of 193 amino acids. B, a Blast2 search of the *B. subtilis* 168 protein data base using Sco1 finds a conserved amino acid core with YpmQ. Cysteines 64 and 68 and histidine 154 are shown.

**Fig. 2. TMPD plate assays of *B. subtilis* strains grown on tryptose blood agar base plates.** A strong purple color indicates the ability to oxidize TMPD and thus the presence of cytochrome c oxidase activity. *B. subtilis* strains 168 and BH170 serve as positive and negative controls. A, lane 1, *B. subtilis* 168; lane 2, BH170; lane 3, BH144; lane 4, BH149; and lane 5, BH191. B, lane 1, *B. subtilis* 168; lane 2, BH170; lane 3, BH150; lane 4, BH193; lane 5, BH183; lane 6, BH148; and lane 7, BH180.

Since ypmQ was identified by its sequence similarity to Sco1 from yeast, and Sco1 is implicated in copper delivery to cytochrome c oxidase, we decided to test whether the deficiency of cytochrome c oxidase that occurs when ypmQ is deleted can be overcome by supplementing the growth medium with copper. We prepared membrane extracts from wild type *B. subtilis*, BH144 (∆*ypmQ*), in which ypmQ is deleted, and BH150 (∆*ypmQ*Δ*ypmR*), in which the *ypmQ* deletion is complemented by expression of *ypmQ* from a plasmid. These three strains were grown under conditions of low copper and are compared with BH144 grown in medium containing high copper (Figs. 3 and 4).

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The histidine-tagged version of ypmQ is equal to the wild type protein in its ability to complement ∆*ypmQ* (Fig. 2B, lanes 1 and 3).

A 6-histidine tag on the functional expression of ypmQ (BH150).

**Table 1.** Relative copper oxidase activity

| Strain | Copper Oxidase Activity Relative to Wild Type |
|--------|---------------------------------------------|
| BH144  | 100%                                        |
| BH150  | 25%                                         |
| BH149  | 0%                                          |

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menaquinol oxidase. The activity of BH150 is about 60% of the wild type activity. The addition of extra copper to the growth medium of BH144 stimulates the activity more than 7-fold over the same strain grown with low copper, which brings it to a level about 25% that seen with the wild type preparation.

Table III also shows the total cytochrome c\(_{aa}\) content for the four conditions mentioned above. In BH144 (\(\Delta ypmQ\)) grown with low copper the cytochrome c\(_{aa}\) content in the extract is reduced to about one-half that seen in the wild type extract. The remaining cytochrome c\(_{aa}\) content is due to the presence of menaquinol oxidase. This change is in keeping with our previous estimate of the cytochrome c oxidase and menaquinol oxidase contents in wild type cells (38) and the loss of cytochrome c oxidase in BH144 (\(\Delta ypmQ\)). When the growth medium is supplemented with copper the cytochrome c\(_{aa}\) content of BH144 is increased to 21% that of wild type. In strain BH150 the level of cytochrome c\(_{aa}\) indicates a recovery to 50% of the wild type level. These cytochrome levels are consistent with the apparent activities, which indicates that to a first approximation the cytochrome c oxidase assembled under these different conditions has close to the same molecular activity. We have also found that the menaquinol oxidase activity is the same in these membrane extracts implying that the lack of YpmQ does not affect its assembly.

In BH144 (\(\Delta ypmQ\)) grown with low copper the spectral properties and activity of the cytochrome c oxidase are absent. Fig. 4 shows the results of an experiment in which antibodies to a highly purified two-subunit form of the cytochrome c\(_{aa}\) complex were used to determine whether the subunits of the enzyme can be detected in this strain. Membrane extracts from wild type B. subtilis, strain BH144 (\(\Delta ypmQ\)), and from BH150 (\(\Delta ypmQ\) \(\Delta ypmQ\)) were run on an SDS gel and transferred to PVDF for Western blotting. The two major subunits, I and II, are clearly observed in the wild type extracts and are both depressed in strain BH144 grown with low copper. Our antibody to cytochrome c\(_{aa}\) has better reactivity with subunit II, and Table III reports the integrated intensity of the subunit II band corrected for the small variation in the amount of membrane protein loaded in each lane of Fig. 4. The level of subunit II is more than 10-fold lower than wild type in BH144 and recovers to more than 80% of wild type levels in BH150 grown with low copper. In BH144 grown with excess copper the subunit II level is 21% of the intensity observed with wild type. The values for subunit II levels are consistent with the cytochrome content and activity levels of these strains (Table III).

A key feature of ypmQ is a set of highly conserved residues that are proposed to function in binding copper. In YpmQ, the cysteines at positions 64 and 68 and histidine at 154 are conserved when compared with the sequence of Sco1 from yeast (39). We expressed ypmQ from a plasmid with amino acids Cys-64 and Cys-68 replaced by serine both individually and together and His-154 changed to alanine. These strains (i.e. BH193, BH183, BH148, and BH180) are not able to oxidize TMPD (Fig. 2B, lane 2) and have a phenotype equivalent to the cytochrome c oxidase disrupted strain (i.e. BH170, Fig. 2B lane 2). Fig. 5 compares the cytochrome content of a B. subtilis strain expressing wild type YpmQ to a strain expressing an inactive mutant of YpmQ (BH180 and H154A). The strain expressing wild type YpmQ has an absorption maximum at 602 nm that is a composite of contributions from the two heme A-containing oxidases (Fig. 5A). In contrast, the strain expressing an inactive mutant of YpmQ has an absorption maximum

Table III

| Strain and copper content | cytochrome c\(_{aa}\) | Ascorbate-TMPD anaerobiosis time | Integrated intensity of subunit II |
|---------------------------|---------------------|---------------------------------|----------------------------------|
| Wild type, low copper     | 2.20                | 24                              | 100                              |
| BH144 (\(\Delta ypmQ\)) low copper | 1.66                | 600                             | 81.5                             |
| BH150 (\(\Delta ypmQ\) (\(\Delta ypmQ\)) low copper | 1.39                | 78                              | 21.0                             |
at 600 nm, which is characteristic of the menaquinol oxidase.
Furthermore, the difference spectrum between the membrane extract of the wild type strain minus the mutant strain shows a deficiency in cytochrome c oxidase (Fig. 5B). The double difference spectrum has a set of bands at 416, 520, and 550 nm due to the cytochrome c domain of subunit II and at 444 and 604 nm due to cytochromes a and a3, which are signatures of B. subtilis cytochrome c oxidase. There is also a difference in the level of cytochrome b in the two strains as evidenced by the peak at 560 nm in the double difference spectrum.

The lack of functional cytochrome c oxidase in the strains expressing site-specific mutants of YpmQ (see Fig. 2B, lanes 4–7) could be due to poor expression or improper folding of the mutant forms of YpmQ. To address this question, we have taken advantage of the histidine tag on each of these constructs and used an anti-histidine tag antibody to detect YpmQ. A Western blot of membrane extracts separated by SDS-PAGE shows that histidine-tagged, native YpmQ and His-tagged, mutant YpmQ proteins are present at similar levels in these different strains (Fig. 6). Since the mutants are expressed to a similar level as wild type and are present in the plasma membrane, it is unlikely that the cysteine to serine or the histidine to alanine changes have compromised the proper expression and folding of these mutant YpmQ proteins.

The effect of ypmQ's deletion on menaquinol oxidase function can also be tested by growth and antibiotic sensitivity. It has been shown that a deficiency in menaquinol oxidase leads to a small colony morphology and confers streptomycin resistance to B. subtilis (21, 40). We have confirmed that a Δpx oxidase strain (BH101) in which the menaquinol oxidase operon is deleted is resistant to streptomycin, whereas the wild type strain and BH144 (ΔypmQ) fail to grow in the presence of streptomycin. We have also observed that BH144 (ΔypmQ) has a colony morphology that is similar to the wild type strain 168 when both are compared with the small colony phenotype observed for BH101 (Δpx oxidase) (data not shown). Spectral evidence, kinetic activity, and growth phenotype support the conclusion that deletion of ypmQ does not affect the expression of menaquinol oxidase.

**DISCUSSION**

Study of the assembly of copper centers in a number of copper-containing proteins has resulted in the identification of specific accessory proteins that aid the assembly process. In some cases transfer of copper from the assembly protein to an apoprotein target has been demonstrated, and these proteins are identified as copper chaperones. This new class of proteins functions to deliver copper and avoids the potential deleterious side effects that could arise from redox reactions initiated by free copper. For example, the copper center in copper, zinc, superoxide dismutase in yeast and humans is assembled with the aid of the copper chaperone protein, CCS (41). In the case of mitochondrial cytochrome c oxidase it has been postulated that the protein Cox17 is responsible for delivering copper from the cytoplasm to the intermembrane space of mitochondria where the metal is incorporated into cytochrome c oxidase (20). Yeast that are deficient in cox17 are unable to grow on non-fermentable substrates but can be rescued by addition of copper to the growth medium (42) or by overexpression of the inner mitochondrial membrane protein, Sco1 (19). In contrast, yeast deficient in Sco1 cannot be rescued by addition of copper to the growth medium. Thus, the working model for copper delivery to cytochrome c oxidase has Cox17-binding copper in the cytoplasm, passing through the outer mitochondrial membrane and delivering copper to the intermembrane space (19). Whether copper is passed from Cox17 to cytochrome c oxidase directly, or with the intervention of Sco1, is not known.

In B. subtilis two homologous members of the cytochrome oxidase family are expressed that have different copper contents. The cytochrome c oxidase complex is most similar to the
mitochondrial enzyme in having both CuA and CuB centers. The menaquinol oxidase does not have CuA but does have the other redox active metals including CuB. When the expression of the Sco1 homolog, YpmQ, is disrupted the spectral properties, major subunits, and activity of cytochrome cox of B. subtilis is lost without affecting the properties of the menaquinol oxidase. This disruption can be overcome partially when extra copper is added to the growth medium and more fully when native YpmQ is expressed from a plasmid. These data lead us to suggest that YpmQ is involved in the assembly of CuB, but is not involved with CuA assembly. Furthermore, we suggest that our results support the proposal that the role of Cox17 in yeast is to deliver copper to the mitochondrial compartment but Sco1 mediates copper incorporation into cytochrome c oxidase. B. subtilis does not, therefore, require a homolog of Cox17 because of its simpler prokaryotic cell structure. However, further work is required to demonstrate the direct role of these proteins in copper delivery to cytochrome c oxidase, and we cannot exclude the presence of an analogous protein to yeast Cox17 being present in B. subtilis.

In our work here it is shown that the failure to produce YpmQ results in a loss of the cytochrome c oxidase complex. Previous work on the related quinone oxidase from E. coli shows that deprivation of copper from the growth medium yields an enzyme that has both the low and high spin heme centers but lacks CuB (43). More recently Hosler and co-workers (44) demonstrate similar effects in cytochrome c oxidase of Rhodobacter sphaeroides when an assembly gene, cox11, is deleted. Deletion of cox11 results in the expression of a cytochrome c oxidase complex that has all the redox metal centers of the native enzyme, including CuB, but lacks CuA. Moreover, based on this work and that on the E. coli oxidase, it appears that it is possible to assemble a CuB-less enzyme and implies that this step occurs late in the assembly of the oxidase complex. In contrast, in our work we do not find a cytochrome c oxidase complex assembled that simply lacks the CuA center, and this implies that CuA assembly occurs at an early stage and is required for subsequent steps or for the overall stability of the complex in vivo.

In a recent paper (45) on mutants of Sco1 from yeast, two conserved cysteine residues, which we have studied in YpmQ, were changed to alanine, and these mutants failed to restore respiratory competence. However, as the authors of this work acknowledge, they cannot exclude that the mutant forms of Sco1 were improperly processed in some way and that this may account for the inactivity. However, we demonstrate that our mutant and wild type versions of YpmQ are equally well expressed and are all found in the plasma membrane. We propose, therefore, that the two conserved cysteine residues and, in addition, the conserved histidine play a direct role in the function of YpmQ. If YpmQ is a homolog of the Sco proteins from yeast and human, then the cysteines and histidine are also critical to their function (30). We have generated histidine-tagged, wild type YpmQ as well as versions in which each of the putative copper ligating residues has been inactivated. We will isolate each of these proteins and investigate their metal-binding properties to clarify further the role proposed above for these residues in copper binding.

The copper chaperone protein, CCS, has considerable homology to its target protein, superoxide dismutase (46). In fact, recent mutagenesis work shows that CCS can be converted to an active superoxide dismutase by a single amino acid change (47). The structural similarity between CCS and superoxide dismutase is proposed to mediate copper exchange via heterodimeric complex formation. In the case of cytochrome c oxidase the copper chaperone Cox17 has been shown to bind two copper ions (48). This could be of significance given that one of its plausible targets, CuA, is a dinuclear copper center, although the geometry of the two sites is different. Sco1 and YpmQ exhibit sequence homology with a small portion (i.e. 20 amino acids) of the CuA domain of cytochrome c oxidases that is centered around the pair of cysteine residues of the copper-binding sites (19). Such limited structural similarities between the CuA site and its possible chaperones, Cox17 and YpmQ or Sco1, do not support a chaperone/target recognition mechanism such as that proposed for the CCS/superoxide dismutase system.

Our results support the proposal that YpmQ is a homolog of the yeast protein Sco1 and that it plays a functional role in the assembly of cytochrome c oxidase in B. subtilis. Deletion of ypmQ disrupts the expression of cytochrome c oxidase but not of menaquinol oxidase. Therefore, we propose that the protein encoded by ypmQ is involved specifically in the assembly of CuA but is not required for the assembly of CuB. This may be a reflection of the different structural features of the CuA and CuB centers. For example, CuB is located in an aqueous-exposed domain about 5 Å from the surface of the protein, whereas CuA is bound to three histidine residues contained within a transmembrane helix of subunit I and is about 15 Å from the closest water-exposed surface of the protein (13, 14).
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Characterization of YpmQ, an Accessory Protein Required for the Expression of Cytochrome c Oxidase in Bacillus subtilis
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