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CtaG is required for formation of active cytochrome *c* oxidase in *Bacillus subtilis*

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The Gram-positive bacterium *Bacillus subtilis* contains two respiratory oxidases of the haem-copper superfamily: cytochrome aa_3, which is a quinol oxidase, and cytochrome caa_3, which is a cytochrome *c* oxidase. Cytochrome *c* oxidase uniquely contains a di-copper centre, Cu_A. *B. subtilis* CtaG is a membrane protein encoded by the same gene cluster as that which encodes the subunits of cytochrome *c* oxidase. The role of *B. subtilis* CtaG and orthologous proteins present in many other Gram-positive bacteria has remained unexplored. The sequence of CtaG is unrelated to that of CtaG/Cox11p of proteobacteria and eukaryotic cells. This study shows that *B. subtilis* CtaG is essential for the formation of active cytochrome caa_3 but is not required for assembly of the core subunits I and II with haem in the membrane and it has no role in the synthesis of active cytochrome aa_3. *B. subtilis* YpmQ, a homologue to Sco1p of eukaryotic cells, is also a membrane-bound cytochrome *c* oxidase-specific assembly factor. Properties of CtaG- and YpmQ-deficient mutants were compared. Cells lacking YpmQ showed a low cytochrome *c* oxidase activity and this defect was suppressed by the supplementation of the growth medium with copper ions. It has previously been proposed that YpmQ/Sco1p is involved in synthesis of the Cu_A centre. The results of this study are consistent with this proposal but the exact role of YpmQ in assembly of cytochrome *c* oxidase remains to be elucidated.

INTRODUCTION

Enzymes belonging to the haem-copper superfamily of respiratory oxidases are found in a broad variety of organisms including *Bacteria*, *Archaea* and *Eukarya*. How these membrane-bound multi-subunit metallo-enzymes are assembled in cells is far from understood. Their biogenesis is apparently complicated, involving many factors, as indicated by studies with the yeast *Saccharomyces cerevisiae* in particular (Tzagoloff & Dieckman, 1990) but also with other micro-organisms (Thöny-Meyer, 1997). Very little is known about the synthesis of haem-copper oxidases in Gram-positive bacteria such as *Bacillus subtilis*.

B. subtilis cells grown under oxic conditions contain several terminal respiratory oxidases (von Wachenfeldt & Hederstedt, 1992, 2002). Two of these are *a*-type cytochromes. Cytochrome caa_3 is a cytochrome *c* oxidase with four polypeptide subunits encoded by the *ctaCDEF* gene cluster (van der Oost *et al.*, 1991; Saraste *et al.*, 1991). The closely related enzyme cytochrome aa_3 is a quinol oxidase with four subunits encoded by the *qoxABCD* operon (Santana *et al.*, 1992; Lemma *et al.*, 1993). Subunit I of these oxidases (CtaD and QoxA) contains two haem A groups, haem *a* and haem a_3, and one copper atom designated Cu_B. Haem a_3 and Cu_B form a binuclear centre where dioxygen is reduced to water (Powers *et al.*, 1994). The electrons required for the reaction enter the binuclear centre from haem *a* (Michel *et al.*, 1998). Subunits II of the two enzymes (CtaC and QoxB) are related proteins but there are major differences. Processed CtaC is a lipoprotein with a membrane-integral N-terminal domain, having two transmembrane α-helical segments, and a C-terminal peripheral domain exposed on the positive (outer) side of the cytoplasmic membrane (Bengtsson *et al.*, 1999). The peripheral domain contains two subdomains: one with a di-copper centre, Cu_A, the other being a mono-haem cytochrome *c* (van der Oost *et al.*, 1991; von Wachenfeldt *et al.*, 1994). Electrons enter the enzyme at the cytochrome *c* and are transferred via the Cu_A centre to haem *a*. QoxB of cytochrome aa_3 is also a lipoprotein (J. Bengtsson & C. von Wachenfeldt, unpublished data) but contains no haem or copper centers. Subunits III (CtaE and QoxC) and subunits IVB (CtaF and QoxD) contain no known prosthetic groups. Isolated active

Abbreviations: ICP-MS, inductively coupled plasma emission mass spectroscopy; TMPD, *N,N,N′N′*-tetramethyl-*p*-phenylenediamine.

preparations of the *B. subtilis* oxidases often lack one or both of subunits III and IVB (cf. Lemma *et al.*, 1993).

B. subtilis cells offer several experimental advantages in a genetic-based approach to find and identify factors involved in assembly of oxidases. Cytochrome caa_3 and cytochrome aa_3 are not essential for growth because their basic function can be replaced by that of cytochrome *bd* (Winstedt & von Wachenfeldt, 2000). Presence of functional cytochrome caa_3 can be specifically detected *in vivo* using the redox dye *N,N,N′N′*-tetramethyl-*p*-phenylenediamine (TMPD) (van der Oost *et al.*, 1991). Colonies containing cytochrome *c* oxidase activity turn blue in the presence of TMPD. After random or site-specific mutagenesis, colonies with TMPD-oxidation-deficient cells can easily be detected on agar plates. From light absorption spectra of membranes it can then be determined if the mutants are defective in for example cytochrome *c* synthesis (*B. subtilis* contains four different cytochromes *c*) (Schiött *et al.*, 1997a), or in haem A synthesis (such mutants lack both cytochrome aa_3 and cytochrome caa_3) (Svensson *et al.*, 1993). Isolated TMPD-oxidation-deficient mutants that contain cytochrome aa_3 might carry mutations in the *ctaCDEF* gene cluster or in genes encoding assembly factors specific for cytochrome caa_3. Mattatall *et al.* (2000) have shown that the YpmQ protein plays a role in assembly of cytochrome *c* oxidase in *B. subtilis*. YpmQ is a predicted lipoprotein of 177 amino acid residues exposed on the positive side of the cytoplasmic membrane.

We have in this work investigated the role of CtaG in *B. subtilis* and have identified *ctaG* and *ypmQ* mutations by screening mutant libraries for cytochrome caa_3-deficient clones. The *ctaG* gene in the *ctaBCDEFG* cluster was found and named in the *B. subtilis* genome sequence project (Kunst *et al.*, 1997) but the functional importance of the gene or the protein it encodes has not previously been addressed. CtaG is a predicted 297 amino acid residue protein with seven α-helical transmembrane segments and the N-terminus exposed on the positive side of the cytoplasmic membrane. It should be noted that *B. subtilis* CtaG is not similar to the CtaG proteins of *Paracoccus denitrificans* and *Rhodobacter sphaeroides*, which are homologous to Cox11p of yeast, and function in the formation of the Cu_B centre (Hiser *et al.*, 2000; Carr *et al.*, 2002). We have analysed CtaG- and YpmQ-deficient strains in parallel and compared their molecular properties.

METHODS

Strains and plasmids. Bacterial strains and plasmids used in this work are presented in Table 1.

Growth media. *Escherichia coli* strains were grown on tryptose blood agar base (TBAB; Difco) plates or in LB (Sambrook *et al.*, 1989). *B. subtilis* strains were grown on TBAB plates, in tryptose broth (TB; contains, per litre, 10 g tryptose, 3 g beef extract and 5 g NaCl) or in nutrient sporulation medium with phosphate (NSMP) (Fortnagel & Freese, 1968). For *E. coli*, the growth media were supplemented with 15 μg tetracycline ml^{-1} or 100 μg ampicillin ml^{-1}. For *B. subtilis* the following concentrations of antibiotics were used: tetracycline, 15 μg ml^{-1}; spectinomycin, 100–300 μg ml^{-1}; kanamycin, 5 μg ml^{-1}; chloramphenicol, 5 μg ml^{-1}. Copper was added to media in the form of $CuCl_2$ dissolved in water.

Molecular genetic techniques. General molecular genetic techniques were as described by Sambrook *et al.* (1989). Plasmid DNA was isolated using CsCl density-gradient centrifugation (Ish-Horowicz & Burke, 1981) or by using the Quantum Prep mini preparation kit (Bio-Rad). Chromosomal DNA from *B. subtilis* strains was isolated as described by Marmur (1961). Transformation of *B. subtilis* using natural competence was done as described by Hoch (1991). *E. coli* cells were transformed by electroporation as before (Le Brun *et al.*, 2000). PCR was performed using the Expand High Fidelity PCR kit (Roche Molecular Biochemicals).

Fluorescent DNA sequencing was carried out on isolated plasmid DNA or PCR products using the BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems) and an ABI prism 3100 DNA sequencer (PE Biosystems).

Construction of plasmids. To obtain plasmid pCTAG1 (Fig. 1), a 544 bp DNA fragment was amplified using PCR with *B. subtilis* 1A1 chromosomal DNA as template and the primers CtaG1 (5′-GGGAATTCAGCTCGGAGCAGTCATGATGA-3′) and CtaG11 (5′-GGGTCGACAGTGTTGAAGTTTACTGAAAGCG-3′). The DNA product was cut with *Eco*RI and *Sal*I (sites underlined in the primer sequences) and ligated into *Eco*RI/*Sal*I-cut pDG1515. Plasmid CTAG2 (Fig. 1) was constructed by ligating a *Xba*I/*Bam*HI-cut 356 bp PCR fragment obtained by using 1A1 chromosomal DNA and primers CtaG13 (5′-GCTCTAGACGTGGCATTTGGGCTTATG-TGG-3′) and CtaG9 (5′-GGGGATCCATCTCCAACTGATTCATT-TCTTCACC-3′) into *Xba*I/*Bam*HI-cut pCTAG1. Plasmid pCTAG5 contains the entire *ctaG* gene under control of the IPTG-inducible *spac* promoter. This plasmid was constructed by ligating the PCR fragment obtained using primers CtaG16 (5′-GGCCGTCGACT-GATTCAGGACATAGCCAACATG-3′) and CtaG17 (5′-CCCAAGC-TTGGAGGTGAAGAAATGAATCAGTTGG-3′) into *Hin*dII/*Sal*I-cut pDG148.

The plasmid pYPMABspc (Fig. 2) was constructed as follows. The 331 bp PCR product obtained using 1A1 DNA and primers YpmQ1 (5′-GTTACGGATCCATTTGCAATTCTACT-3′) and YpmQ2 (5′-GCCCTGCAGTTAACCCCTTGAT-3′) was cut with *Bam*HI and *Pst*I and ligated into *Bam*HI/*Pst*I-cut pBluescript SK. The plasmid obtained was cut with *Pst*I and *Xho*I and ligated to the *Pst*I/*Xho*I-cut 388 bp PCR fragment obtained by PCR using primers YpmQ3 (5′-TCAGC-CTGCAGACTCAAGTAAGC-3′) and YpmQ4 (5′-GTTTCTCGAG-TAAATCATCCGAACG-3′). The resulting plasmid was cut with *Pst*I and ligated to a 1194 bp *Pst*I fragment from pSPC1 containing the *spc* gene. Plasmid pYPMQ1 (Fig. 2) was constructed by ligating the *Bam*HI/*Xho*I-cut 1251 bp PCR product resulting from amplification using the primers YpmQ1 and YpmQ4 into pHPSK.

The final step in the construction of plasmids CTAG5 and pYPMQ1, i.e. transformation with ligate, was done using *B. subtilis* strain 1A1 to avoid problems with toxicity of the plasmids in *E. coli*. All cloned PCR products were confirmed by DNA sequence analysis. It should be noted that the published *ypmQ* sequence available in databases has an error (Mattatall *et al.*, 2000; our own sequence data): one G residue should be an A and this results in a glutamate residue at position 85 in YpmQ (the incorrect sequence predicts a glycine residue).

Construction of strains. *B. subtilis* strain LJB107 was obtained by transforming strain 1A1 with pYPMABspc, made linear by digestion with *Sca*I, and selecting for spectinomycin-resistant clones. Strain LUW202 resulted from the transformation of 1A1 with pCTAG2. Deletion of genes in strains was confirmed by using PCR with isolated

Table 1. Strains and plasmids used in this work

Strain	Relevant properties*	Source†	Reference
1A1	*trpC2*		BGSC‡
BFS2217	*ypmQ*ΩpMUTIN2		P. Nygaard
CMW19	*trpC2* Δ*cydABCD*::*tet ypmQ19*	EMS mutagenesis of LUW35	Le Brun *et al.* (2000); this work
CMW24	*trpC2* Δ*cydABCD*::*tet ctaG24*	EMS mutagenesis of LUW35	Le Brun *et al.* (2000); this work
LJB107	*trpC2* Δ*ypmQ*::*spc*	pYPMABspc→1A1	This work
LJB108	*trpC2* Δ*ypmQ*::*spc* Δ*ctaG*::*tet*	LUW202→LJB107	This work
LJB109R	*trpC2* Δ*ctaG*::*tet* Δ*qoxABCD*::*kan*	LUH14→LUW202	This work
LJB110R	*trpC2* Δ*ypmQ*::*spc* Δ*qoxABCD*::*kan*	LUH14→LJB107	This work
LJB111	*trpC2* Δ*ypmQ*::*spc* Δ*ctaG*::*tet* Δ*qoxABCD*::*kan*	LUH14→LJB108	This work
LUH14	*trpC2* Δ*qoxABCD*::*kan*		L. Hederstedt
LUT3	*trpC2* Δ*ctaCD*::*ble*	JO1→1A1	T. Schiött & L. Hederstedt
LUW20	*trpC2* Δ*cydABCD*::*tet*		Winstedt *et al.* (1998)
LUW34	*trpC2* Δ*cydABCD*::*cat*		Winstedt & von Wachenfeldt (2000)
LUW35	*trpC2* Δ*cydABCD*::*tet*	LUW20→1A1	This work
LUW46	*trpC2* Δ*qoxABCD*::*kan*		Winstedt & von Wachenfeldt (2000)
LUW143R	*trpC2* Δ*qoxABCD*::*kan* Δ*ctaCD*::*ble*		Winstedt & von Wachenfeldt (2000); this work
LUW202	*trpC2* Δ*ctaG*::*tet*	pCTAG2→1A1	This work
LUW207	*trpC2* Δ*cydABCD*::*cat* Δ*ypmQ*::*spc*	LJB107→LUW34	This work
LUW208	*trpC2*Δ*cydABCD*::*cat* Δ*ctaG*::*tet*	LUW202→LUW34	This work
ORE1	*trpC2 ypmQ*ΩpMUTIN2	BFS2217→1A1	This work
Plasmids			
pYPMQ1	*ypmQ* on a 1251 bp *Bam*HI/*Xho*I fragment in pHPSK; Cap Erm (Fig. 2)		This work
pYPMABspc	Δ*ypmQ*::*spc* in pBluescript; Amp Spc (Fig. 2)		This work
pDG148	P*spac lacI*; Amp Kan		Stragier *et al.* (1988)
pDG1515	Tet Amp		Guérout-Fleury *et al.* (1995)
pBluescript II SK(−)	Amp		Stratagene
pCTAG1	Tet Amp (Fig. 1)		This work
pCTAG2	Tet Amp (Fig. 1)		This work
pCTAG5	*ctaG* on a *Hin*dIII/*Sal*I fragment under the P*spac* promoter in pDG148; Kan (Fig. 1)		This work
pSPC1	pBluescript containing the *spc* gene of pDG1726 on a *Sma*I/*Eco*RV fragment; Amp Spc		M. Throne-Holst, Lund University
pHPSK	Erm Cap		Johansson & Hederstedt (1999)

*Tet, Spc, Kan, Amp, Cap and Erm indicate resistance to tetracycline, spectinomycin, kanamycin, ampicillin, chloramphenicol and erythromycin, respectively.

†Plasmid or chromosomal DNA indicated to the left of the arrow was used in transformation of the strain indicated.

‡*Bacillus* Genetic Stock Center, Ohio, USA.

chromosomal DNA. Strains LJB109R and LJB110R are isolates that in addition to the introduced deletion-substitutions (Table 1) contain spontaneous, unidentified, mutations that allow these strains to grow better than the original isolates on TBAB plates. LUW143R is also such a better-growing isolate of strain LUW143. The unknown mutations result in the expression of *cydABCD* also when cells are grown in the absence of glucose in the medium.

Analysis of TMPD-oxidation activity. Colonies were either stained for TMPD-oxidation activity directly on agar plates as described before (Le Brun *et al.*, 2000), or (in the case of agar plates where the medium had been supplemented with transition metal ions) the colonies were grown on a 0·45 μm pore size filter (Millipore HATF surfactant free) laid on top of the agar. After incubation of the plate overnight the filter was rinsed in water and then stained using the same mixture as for plates except that the agar solution was replaced with a corresponding volume of water.

Quantification of CtaC and CtaD by immunoblot analysis. SDS-PAGE was performed according to Schägger & von Jagow (1987). The proteins were then transferred to a PVDF membrane (Immobilon-P, Millipore) in a wet blot using the following

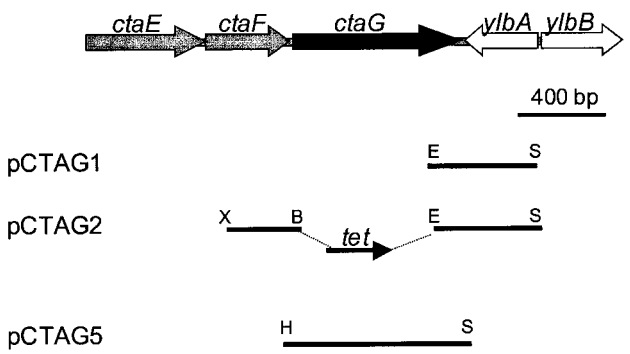

Fig. 1. Map of the *ctaG* gene and flanking genes in the *B. subtilis* chromosome. DNA fragments from the *cta* region cloned into plasmids pCTAG1, pCTAG2 and pCTAG5 are shown. Sites for restriction enzymes are indicated: B, *Bam*HI; H, *Hin*dIII; E, *Eco*RI; S, *Sal*I; X, *Xba*I. The tetracycline resistance gene is indicated by *tet*.

electrophoresis buffer: 2·4 g Tris l^{-1}, 11·25 g glycine l^{-1} and 20 % (v/v) methanol. The anti-CtaC rabbit serum used recognizes the C-terminal part of *B. subtilis* CtaC and has been described before (Bengtsson *et al.*, 1999). Anti-CtaD serum was obtained by immunizing rabbits with a synthetic peptide (CHIHKEELPNDDKGVKA) conjugated to keyhole limpet haemocyanin. The peptide sequence, except for the cysteinyl residue, corresponds to the 16 most C-terminal residues of *B. subtilis* CtaD. The secondary antibody was horseradish-peroxidase-conjugated anti-rabbit antibodies. Bound secondary antibodies were detected using the Super Signal reagent (Pierce). Chemiluminescence was recorded using a Kodak Image Station 440CF and relative intensities were analysed using the Kodak 1 D Image Analysis Software, version 3.0.

Miscellaneous methods. Liquid cultures of *B. subtilis* were grown in batches of 1 litre in 5 litre Erlenmeyer flasks with indentations. The cultures were incubated at 37 °C on a rotary shaker (200 r.p.m.) and growth was followed by measurement of OD_{600}. Membranes were isolated as described by Hederstedt (1986). Protein concentrations were estimated using the BCA method (Pierce) with bovine serum albumin as the standard. Cytochrome *c* oxidase activity measurements using reduced *S. cerevisiae* cytochrome *c* (Sigma) were performed as described before (van der Oost *et al.*, 1991). Light absorption spectroscopy at room temperature was performed as described previously (Schiött *et al.*, 1997a).

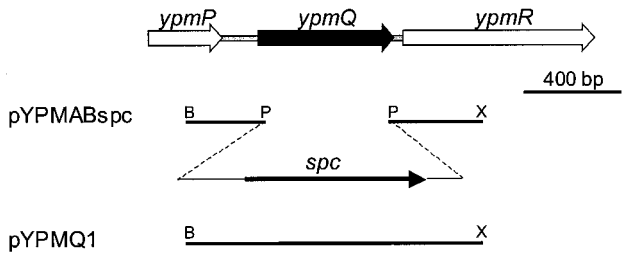

Fig. 2. Map of the *ypmQ* gene and flanking genes in the *B. subtilis* chromosome. DNA fragments present in plasmid pYPMABspc (used for deletion of the *ypmQ* gene) and plasmid pYPMQ1 (used for overexpression of *ypmQ*) are shown. Restriction sites are indicated: B, *Bam*HI; P, *Pst*I; X, *Xho*I. The spectinomycin-resistance gene is indicated by *spc*.

RESULTS

B. subtilis CtaG is required for cytochrome caa_3 activity

To determine if the CtaG protein is important for cytochrome caa_3 synthesis or activity we deleted the *ctaG* gene in the chromosome of strain 1A1 and replaced it with a gene encoding tetracycline resistance (Fig. 1). Colonies of the resulting strain, LUW202, on TBAB plates lacked TMPD-oxidation activity. LUW202 containing plasmid pCTAG5, carrying the *ctaG* gene (Fig. 1), showed TMPD-oxidation activity on TBAB plates, i.e. the plasmid complemented the defect in the chromosome. LUW202 containing only the plasmid vector, pDG148, was TMPD-oxidation negative, as expected. In pCTAG5, the *B. subtilis ctaG* gene is transcribed from the *spac* promoter. This recombinant promoter is controlled by the LacI protein, in this case encoded by the plasmid, and is inducible with IPTG. A low level of promoter activity is seen in the absence of the inducer compound. In accordance with this property, colonies of LUW202/pCTAG5 on TBAB plates showed a more intensive TMPD-oxidation activity if the medium was supplemented with 50 μM IPTG compared to unsupplemented plates.

In previous work we have, after mutagenesis of spores using ethyl methane sulfonate (EMS), isolated a collection of mutants deficient in TMPD-oxidation activity when grown on TBAB plates (Le Brun *et al.*, 2000; Bengtsson, 2001). Some of these mutants are blocked in cytochrome *c* synthesis. As a result of this defect the cytochrome *c* domain of cytochrome caa_3 is not assembled and the mutants therefore lack cytochrome *c* oxidase activity (Le Brun *et al.*, 2000). Twenty-four mutants from this collection which were known to contain cytochrome *c* were transformed with pCTAG5 and pDG148 and then assayed for TMPD-oxidation activity. One mutant strain, CMW24, was complemented by pCTAG5 but not by pDG148. DNA sequence analysis of the *ctaG* gene in the chromosome of CMW24 showed a nonsense mutation (C to T transition creating a stop codon at position 261 in CtaG). The resulting predicted CtaG protein is truncated, lacking the most C-terminal transmembrane segment (residues 261–297). The findings demonstrate that the CtaG protein is important for cytochrome caa_3 activity in *B. subtilis*.

YpmQ-deficient mutants

As part of the systematic *B. subtilis* Gene Function Analysis Project (Schumann *et al.*, 2001) the plasmid pMUTIN2 was inserted at various known positions in the *ypmPQRST* gene cluster located at 195° on the chromosomal genetic map (Fig. 2). Except for YpmQ, the functions of the proteins

encoded by the *ypmQRST* gene cluster are largely unknown. Colonies of strain BFS2217, which has the *ypmQ* gene disrupted, on TBAB plates were found to be deficient in TMPD-oxidation activity. Strains BSF2218, BSF2219 and BSF2220, with various insertions in the operon downstream of the *ypmQ* gene, showed wild-type TMPD-oxidation activity. To work with isogenic strains, the pMUTIN2-insertion in the *ypmQ* gene was moved from BSF2217 to strain 1A1, resulting in strain ORE1. Colonies of ORE1 on TBAB plates were TMPD-oxidation negative as expected.

A promoter region in front of the *ypmQ* gene has tentatively been identified (Mattatall *et al.*, 2000). Based on expression of *lacZ* from the integrated pMUTIN2, the *ypmQ* promoter activity was found to be repressed by the presence of glucose in the medium and showed a peak at the end of the exponential growth phase (data not shown). This pattern is reminiscent of that for *ctaC* gene expression (Liu & Taber, 1998).

We deleted the *ypmQ* gene in strain 1A1 and replaced it with a gene encoding spectinomycin resistance (Fig. 2). Colonies of the resulting mutant strain, LJB107, transformed with plasmid pYPMQ1 (carrying *ypmQ* and its natural promoter region) were TMPD-oxidation positive on TBAB plates. LJB107 containing only the plasmid vector, pHPSK, was deficient in TMPD-oxidation activity. Plasmids pYPMQ1 and pHPSK were also used to transform the collection of 24 mutants with EMS-induced mutations. Strain CMW19 was complemented by pYPMQ1 but not pHPSK. Sequence analysis of chromosomal DNA from CMW19 showed a G to A substitution within the *ypmQ* gene, changing residue Cys-68 to Tyr in the YpmQ protein.

YpmQ-deficient mutants show a leaky phenotype

It was observed that strains deficient in YpmQ showed TMPD-oxidation activity when grown on NSMP plates but lacked such activity when grown on TBAB plates. CtaG-deficient mutants, in contrast, showed the same TMPD-negative phenotype on both NSMP and TBAB plates. Expression of the structural genes for cytochrome caa_3 (*ctaCDEF*) in *B. subtilis* is under catabolite repression and therefore dependent on the composition of the growth medium (Liu & Taber, 1998). The observed difference in TMPD-oxidation activity of the YpmQ-deficient mutants could therefore be due to different cellular levels of functional cytochrome caa_3 depending on the growth medium used.

To test this explanation, we grew the parental strain 1A1 and the YpmQ-deficient mutant ORE1 in liquid TB (equivalent to TBAB) and in NSMP. As a control, we also grew strain LUT3, which completely lacks cytochrome caa_3 because the *ctaCD* genes are deleted from the chromosome. The cultures were harvested about 1 h after the end of the exponential growth phase. At this growth stage the cellular level of cytochrome caa_3 is at its maximum. Cytochrome *c* oxidase activity of membranes isolated from the cultured cells is presented in Table 2. The analysis demonstrated that a YpmQ-deficient mutant contains only 10–15 % of the normal level of cytochrome *c* oxidase activity. The parental strain, 1A1, grown in NSMP contained a fivefold higher enzyme activity than cells grown in TB. This difference was also found in the levels of CtaC protein in the membranes (immunoblot not shown). Membranes from strains 1A1 and ORE1 grown in NSMP supplemented with 0·5 % (w/v) glucose (Table 2) contained only 10–30 % of the cytochrome *c* oxidase activity present in membranes from cells grown in NSMP. This showed that expression of cytochrome caa_3 activity in the parental strain and the YpmQ-deficient strain is under catabolite repression to about the same extent.

Table 2. Cytochrome *c* oxidase activity of membranes isolated from *B. subtilis* strains grown in various media

Strain	Relevant property	Growth medium	Medium supplement	Relative activity*
1A1	Parental strain	NSMP	–	1
		NSMP	0·5 % glucose	0·1
		TB	–	0·2
ORE1	YpmQ$^-$	NSMP	–	0·1
		NSMP	0·5 % glucose	0·03
		TB	–	0·03
LUT3	CtaCD$^-$	NSMP	–	0·01

*The cytochrome *c* oxidase activity of membranes isolated from strain 1A1 grown in NSMP was 780 nmol cytochrome *c* oxidized min^{-1} (mg membrane protein)$^{-1}$. The relative activities presented are the mean values from two independent experiments; variation in results was 11 % or less. Note that the background (LUT3 membranes) activity was 0·01 and this has not been subtracted from the values for 1A1 and ORE1.

The results provided an explanation for the difference in apparent TMPD-oxidation activity of the YpmQ-deficient mutants on TBAB and NSMP plates. In colonies of such mutants grown on TBAB plates the amount of cytochrome caa_3 activity is too low to be detectable using the TMPD staining procedure. NSMP was used as growth medium in all subsequent experiments to optimize expression of cytochrome caa_3 in the strains.

Cytochrome *c* oxidase activity of complemented mutants

The cytochrome *c* oxidase activities of membranes isolated from LUW202/pCTAG5 and LJB107/pYPMQ1 grown in NSMP are shown in Fig. 3. Strain LJB107 was almost completely complemented by the *ypmQ* gene with its

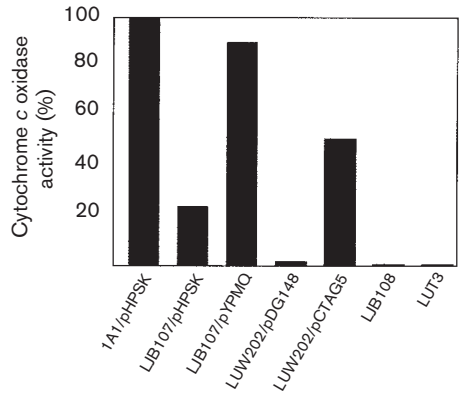

Fig. 3. Cytochrome *c* oxidase activity of membranes isolated from various strains. The strains are described in Table 1. Membranes were isolated from cells grown to early stationary phase in NSMP supplemented with 50 μM IPTG. The relative activities presented are from a single experiment or the mean value from two experiments; 100 % activity of 1A1/pHPSK membranes was 775 nmol cytochrome *c* oxidized min^{-1} (mg membrane protein)$^{-1}$.

natural promoter on the plasmid. LUW202, deleted for the *ctaG* gene, was only complemented to about 50 % by pCTAG5, although the cells were grown in the presence of IPTG to completely induce transcription of *ctaG* on the plasmid. Transcription of the plasmid-borne *ctaG* gene from the recombinant *spac* promoter thus appears to limit the expression of cytochrome *c* oxidase activity in strain LUW202/pCTAG5.

Cytochrome *caa*$_3$ is enzymically defective in CtaG- and YpmQ-deficient strains

Immunoblots with antibodies recognizing the very C-terminal part of the CtaC polypeptide (subunit II of cytochrome *caa*$_3$) were used to analyse enzyme protein contents in YpmQ- and CtaG-deficient mutants. Membranes isolated from strains LJB107 (Δ*ypmQ*) and LUW202 (Δ*ctaG*) grown in NSMP contained 85–105 % and 76–84 %, respectively, of the amount of CtaC present in membranes of the parental strain 1A1.

Upon SDS-PAGE, the CtaC and CtaD polypeptides of YpmQ- and CtaG-deficient mutants and those of the wild-type migrated identically as determined by the immunoblot experiments. This indicated normal post-translational processing of the CtaC polypeptide in the mutants, e.g. diacylglyceride attachment, cleavage of the signal peptide and covalent attachment of haem (Bengtsson *et al.*, 1999).

The observed near-normal amounts of CtaC polypeptide with normal molecular properties in the mutants were also consistent with our unpublished data available for strains CMW19 (*ypmQ19*) and CMW24 (*ctaG24*). These two strains and the parental strain LUW35 grown in NSMP contain normal amounts of CtaC polypeptides as determined by SDS-PAGE and autoradiography of membranes isolated from cells grown in the presence of [^{14}C]aminolaevulinic acid (Le Brun *et al.*, 2000; Bengtsson, 2001). Growth of *B. subtilis* in the presence of this radioactive compound specifically labels haem, and only proteins with covalently bound haem appear as radioactive bands after SDS-PAGE (Schiött *et al.*, 1997b). Using this procedure the relative concentrations of different *c*-type cytochromes in the sample are determined very accurately since all haem groups have the same specific radioactivity.

Cytochrome *a* in mutants

The results obtained using immunoblotting and [^{14}C]haem-labelled cytochromes showed that CtaC polypeptide containing cytochrome *c* and of normal size is present in near-normal amounts in membranes of YpmQ- and CtaG-deficient mutants. This, however, does not provide information on whether subunit I contains haem A.

Fig. 4 shows light absorption difference (reduced minus oxidized) spectra of membranes isolated from strains 1A1, LJB107, LUW202, LJB108 and LUT3 grown in NSMP. Addition of ascorbate to membranes causes preferential reduction of *c*-type cytochromes and cytochrome *a* of cytochrome *caa*$_3$ (Fig. 4a). Consistently, the parental strain 1A1 showed an absorption peak at 605 nm due to cytochrome *a*. Strain LUT3, which lacks cytochrome *caa*$_3$, showed no peak at this wavelength, as expected. The *c*-type cytochromes, which show an absorption maximum at about 550 nm, were present in similar amounts in

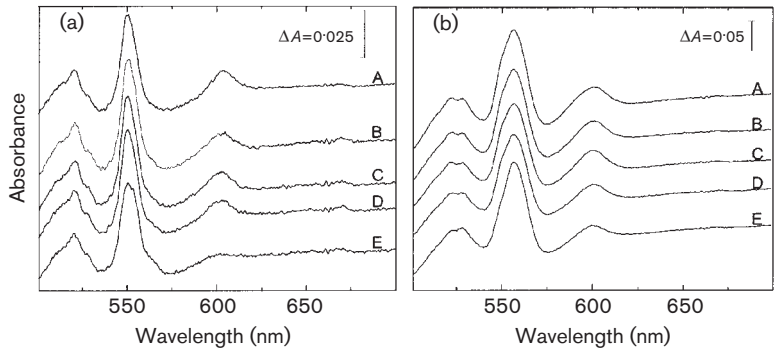

Fig. 4. Light absorption difference (reduced minus oxidized) spectra of membranes isolated from different *B. subtilis* strains grown in NSMP. A, 1A1; B, LJB107; C, LUW202; D, LJB108; E, LUT3. The strains are described in Table 1. (a) Spectra of ascorbate-reduced minus ferricyanide-oxidized membranes. (b) Spectra of dithionite-reduced minus ferricyanide-oxidized membranes. The protein concentration was 3 mg ml^{-1}.

membranes from all strains. The YpmQ- and CtaG-deficient mutants LJB107 and LUW202, respectively, and LJB108, deficient in both YpmQ and CtaG, contained close to normal amounts of ascorbate-reducible cytochrome *a*. Reduction with dithionite, which reduces all *B. subtilis* membrane-bound cytochromes, showed that the overall cytochrome content is unaffected in YpmQ- and CtaG-deficient mutants (Fig. 4b).

The absorption spectra of haem *a* in cytochrome aa_3 and cytochrome caa_3 overlap considerably in the 600 nm region. To remove the spectral contribution from cytochrome aa_3 we deleted the *qoxABCD* operon in YpmQ- and CtaG-deficient strains. *B. subtilis* cells completely lacking cytochrome *a* are viable because they can use the cytochrome *bd* quinol oxidase for respiration. Dithionite-reduced membranes of strain LUW143R (Δ*qoxABCD* Δ*ctaCD*) showed no absorption peak at 600 nm, as expected from the complete lack of *a*-type cytochromes in this strain. The corresponding spectra of strains LJB110R (Δ*ypmQ* Δ*qoxABCD*) and LJB109R (Δ*ctaG* Δ*qoxABCD*) showed cytochrome *a* absorption spectra very similar to that of the positive control, strain LUW46 (Δ*qoxABCD*) (Fig. 5). However, the cytochrome *a* absorption peak at 605 nm in the wild-type case was found to be slightly blue shifted in both mutants. A similar shift in wavelength has been observed for incompletely assembled *R. sphaeroides* cytochrome oxidase (Bratton *et al.*, 2000) and for cytochrome *c* oxidase of Sco1p-deficient yeast cells (Dickinson *et al.*, 2000).

The amounts of CtaC and CtaD polypeptides in LJB110R and LJB109R membranes were 40–60 % relative to those in membranes of the parental strain LUW46 (Fig. 6). These relative concentrations of enzyme protein corresponded well with the relative amounts of cytochrome *a* in these strains as determined by light absorption spectroscopy (Fig. 5). Strain LJB109R lacked cytochrome *c* oxidase activity, whereas LJB110R showed only about 14 % activity compared to the parental strain LUW46 (Fig. 6, legend).

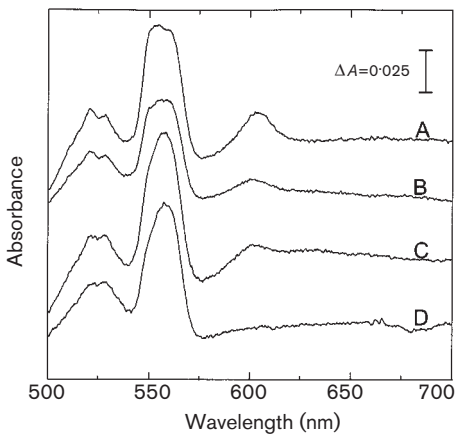

Fig. 5. Light absorption difference (dithionite-reduced minus ferricyanide-oxidized) spectra of isolated membranes from different strains all deleted for *qoxABCD* and therefore lacking cytochrome aa_3. A, LUW46; B, LJB109R; C, LJB110R; D, LUW143R. The strains are described in Table 1. Membranes were isolated from cells grown to early stationary phase in NSMP. The protein concentration was 3 mg ml^{-1}.

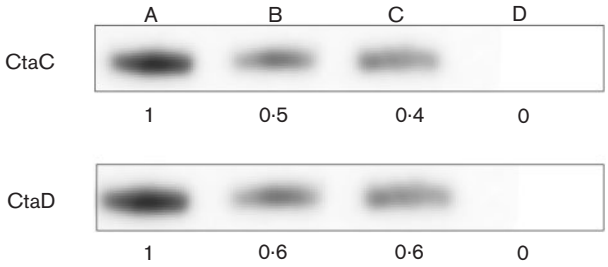

Fig. 6. Western blot analysis of isolated membranes from different *B. subtilis* strains using antisera directed against subunits CtaC and CtaD of cytochrome caa_3. A, LUW46; B, LJB109R; C, LJB110R; D, LUW143R. The membranes analysed were the same as those used for the spectroscopy in Fig. 5. The numbers below each band show the relative amount of antigen. The amount of membrane protein loaded was 5 and 1 μg per lane to detect CtaC and CtaD antigen, respectively. The cytochrome *c* oxidase activities [nmol cytochrome *c* oxidized min^{-1} (mg membrane protein)$^{-1}$] of the membranes were as follows: LUW46, 110; LJB109R, <1; LJB110R, 16; LUW143R, <1.

We conclude that CtaG- and YpmQ-deficient mutants contain defective cytochrome caa_3 in their membranes. The enzyme activity of cytochrome *c* oxidase in CtaG-deficient mutants is very low or absent whereas in YpmQ-deficient mutants it is only 10–20 % of normal.

Cytochrome aa_3 is active in the absence of CtaG and YpmQ

Under oxic conditions, *B. subtilis* cells require a functional cytochrome aa_3 or cytochrome *bd* for growth (Winstedt & von Wachenfeldt, 2000). We exploited this fact to determine if CtaG and YpmQ are specifically required for the synthesis of active cytochrome caa_3 or if they also have a role in synthesis of active cytochrome aa_3. Strains LUW207 and LUW208, deleted for *cydABCD* and the *ypmQ* or *ctaG* gene, were constructed (Table 1). Both these strains grew on TBAB plates like the parental strain only lacking cytochrome *bd*. This result demonstrated that YpmQ and CtaG are not important for assembly of active cytochrome aa_3. Thus, CtaG and YpmQ have no significant role in the assembly of functional quinol oxidases and are not important for general assembly of the haem a_3–Cu_B binuclear centre in the haem-copper oxidases.

The oxidase defect in YpmQ-deficient, but not CtaG-deficient, strains is suppressed by copper in the growth medium

Mattatall *et al.* (2000) showed that copper ions in the growth medium can suppress the phenotype of a

YmpQ-deficient mutant. We found that YpmQ-deficient strains showed a TMPD-oxidation positive phenotype on TBAB plates if 0·5–50 μM $CuCl_2$ was included in the medium. The oxidase defect of CtaG-deficient mutants was in contrast not suppressed by the addition of copper ions to the growth medium.

The YpmQ-deficient strain LJB107 and its parental strain 1A1 were grown in NSMP supplemented with various concentrations of $CuCl_2$. The final total copper concentration in the medium after inoculation with cells was determined by inductively coupled plasma emission mass spectroscopy (ICP-MS). Membranes were isolated from the cells grown to early stationary phase and the cytochrome *c* oxidase activity was analysed (Fig. 7). The activity of LJB107 grown in the presence of 0·5 μM copper was about 15 % compared to strain 1A1 but increased to 50 % at 1·3–4·9 μM copper. The activity of 1A1 also increased somewhat with increasing copper concentrations in the medium but this increase was marginal compared to that observed with LJB107.

Addition of $CoCl_2$ or $MnCl_2$ in the 1–50 μM concentration range to the growth medium had no significant effect on the cytochrome *c* oxidase activity of LJB101 compared to 1A1.

Properties of a CtaG YpmQ double mutant

A CtaG and YpmQ double-deficient strain, LJB108, was constructed (Table 1). The phenotype of this strain was found to be indistinguishable from that of strain LUW202 deleted for the *ctaG* gene only, i.e. isolated membranes of LJB108 lacked cytochrome *c* oxidase activity (Fig. 3), contained ascorbate-reducible cytochrome *a* (Fig. 4), and contained CtaC polypeptide in amounts that were 60 % or more compared to that in membranes from the parental strain 1A1 grown under the same conditions. Addition of $CuCl_2$ to TBAB plates had no effect on the TMPD-oxidation activity of strain LJB108.

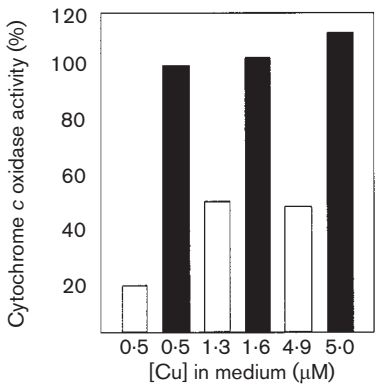

Fig. 7. Cytochrome *c* oxidase activity of membranes isolated from strain LJB107 (Δ*ypmQ*; white bars) and 1A1 (parental strain; black bars) grown in NSMP containing different concentrations of copper; 100 % corresponds to 440 nmol cytochrome *c* oxidized min^{-1} (mg membrane protein)$^{-1}$. The copper concentration in the medium was measured using ICP-MS.

DISCUSSION

In this work we have used a genetic approach to analyse the role of the CtaG and YpmQ proteins in the cytoplasmic membrane of *B. subtilis*. Our results demonstrate that CtaG is required for formation of active cytochrome caa_3. Cytochrome caa_3 subunits I and II polypeptides, of normal sizes and in 40–100 % of the normal concentration, were found in membranes of CtaG-deficient mutants (the relative amount was influenced by the genotype of the reference strain; e.g. membranes of strain 1A1 contained more cytochrome caa_3 than those of LUW46). These membranes also contained haem *a* and haem *c* chromophores of cytochrome caa_3. The cytochrome *a* α-absorption band was found to be blue shifted. By the use of cytochrome *bd*-deficient mutants we show that the lack of CtaG specifically affects cytochrome caa_3, i.e. assembly of functional cytochrome aa_3 does not depend on CtaG. These results combined suggest that CtaG is important for assembly of the Cu_A di-copper centre or some other feature which is uniquely present in the cytochrome caa_3 and is critical for cytochrome *c* oxidase activity of the *B. subtilis* enzyme. The amino acid sequences of *B. subtilis* CtaG and orthologous proteins (COG3336; Tatusov *et al.*, 2001) in other bacteria do not indicate the specific function of the protein but predict an integral membrane protein with seven α-helical transmembrane segments. *Mycobacterium* and *Corynebacterium* species appear to contain a variant of CtaG with an N-terminal extension comprising nine transmembrane segments. Speculatively, CtaG might be involved in transport or signalling across the membrane or might directly act as a kind of chaperone in assembly of the oxidase from its components. The presence of *ctaG* as the last gene in the *ctaBCDEFG* cluster also in other species than *B. subtilis* (e.g. *Geobacillus stearothermophilus* and *Bacillus anthracis*) is logical if the function of CtaG is restricted to cytochrome caa_3.

Interestingly, in the chromosome of *Bacillus halodurans* the *ctaG* gene is located far away from the *ctaCDEF* gene cluster but adjacent to a gene encoding a YpmQ/Sco1p orthologue. Sco1p in yeast has been shown to interact with subunit II of cytochrome oxidase (Lode *et al.*, 2000) and with Cox17p, a copper-binding protein which functions in delivering copper to mitochondria (for a review see Barrientos *et al.*, 2002). Cox17p deficiency results in lack of cytochrome *c* oxidase activity and this can be suppressed by adding copper to the growth medium or by overexpression of *SCO1*. Sco1p deficiency in eukaryotic cells specifically causes cytochrome *c* oxidase deficiency. Humans contain two YpmQ orthologues, SCO1 and SCO2, and the cytochrome oxidase deficiency of SCO1-deficient cells in culture can be suppressed by the addition of copper (Salviati *et al.*, 2002). Sco1p, YpmQ and their homologues,

e.g. PrrC in *R. sphaeroides*, contain two conserved cysteine residues, in a CxxxC motif, and also a conserved histidine residue. Sco1p and PrrC can bind one Cu(I) per monomer and using site-specific mutagenesis the conserved cysteine and histidine residues have been demonstrated to be important for both function and copper binding (Rentzsch *et al.*, 1999; Mattatall *et al.*, 2000; Nittis *et al.*, 2001; McEwan *et al.*, 2002). The available data from studies in yeast suggest that Cox17p delivers Cu(I) to Sco1p, which functions in the assembly of the Cu_A centre (Beers *et al.*, 2002; Punter & Glerum, 2003).

Based on the complete genome sequence, *B. subtilis* apparently does not contain a Cox17p orthologue. The functions of CtaG and YpmQ seem related, i.e. both play a role in assembly of active cytochrome caa_3, but only in YpmQ-deficient mutants is the deficiency in cytochrome *c* oxidase activity incomplete and suppressed by increased copper concentration in the medium. A CtaG YpmQ double-deficient mutant showed the same properties as a mutant lacking only CtaG. These results suggest that CtaG protein has a more fundamental role than YpmQ in the formation of active cytochrome caa_3. Alternatively, there is some other protein(s) in *B. subtilis* that in its function partially overlaps with YpmQ and is affected by copper ions in the medium. The low oxidase activity per molecule of cytochrome caa_3 in YpmQ-deficient strains was found to be about the same also when the amount of enzyme was varied 10-fold (Table 2 and immunoblots not shown).

Mattatall *et al.* (2000) originally demonstrated that YpmQ has a role in synthesis of cytochrome caa_3 in *B. subtilis*. Using other types of experiments than we have exploited here, they concluded that cytochrome caa_3 is specifically affected by a lack of YpmQ. They showed that the phenotype of a strain deleted for the *ypmQ* gene is dependent on the copper concentration in the growth medium. The two conserved cysteine residues (Cys-64 and Cys-68 in *B. subtilis*) and the conserved histidine residue (His-154) in YpmQ were by alanine substitutions shown to be essential for function of the protein. We show here that a replacement of Cys-68 by tyrosine also inactivates YpmQ. From the phenotype of YpmQ-deficient cells, the effect of copper, the sequence similarity of YpmQ to *S. cerevisiae* ScoIp, and the result of mutating the conserved residues in YpmQ, Mattatall *et al.* (2000) suggested that the indicated two cysteine residues and the histidine residue in YpmQ 'are involved in copper exchange between YpmQ and the Cu_A site of cytochrome *c* oxidase'. Our results fully agree with this conclusion but there is one notable inconsistency in our experimental data. We found 40–100 % of the normal amount of CtaC and CtaD polypeptides in membranes of various YpmQ-deficient strains whereas they found only about 10 % of the wild-type level of CtaC after growth at low copper and about 21 % after growth at high copper concentration. This difference is important for the interpretation of the defect caused by YpmQ deficiency and may be explained by differences between strains, growth conditions and membrane isolation procedures. For example, it is known that the cytochrome *c* domains in *B. subtilis* membranes are prone to being 'shawed' off by the action of proteases (von Wachenfeldt & Hederstedt, 1993; Yu *et al.*, 1995) and this affects apparent CtaC concentrations in isolated membranes determined using immunoblotting.

In conclusion, YpmQ in *B. subtilis* and other Gram-positive bacteria might function in delivering copper ions to the Cu_A site in subunit II on the positive side of the cytoplasmic membrane. This suggested direct or indirect role is of major importance only when the availability of copper is low, i.e. when the concentration of copper ions in the surrounding medium is low (less than 1 μM). High copper concentrations in the medium suppress the phenotype of YpmQ-deficient mutants. Hence, YpmQ might act as a periplasmic copper-binding protein helping to capture ions from the medium. It then remains unexplained why YpmQ is not required also for Cu_B assembly and why YpmQ orthologues are present in bacteria that apparently lack Cu_A centre-containing oxidases (Seib *et al.*, 2003). It has been suggested that YpmQ and similar proteins might be thiol–disulfide oxidoreductases rather than metal-binding proteins (Chinenov, 2000). This could explain the Cu_A specificity because two cysteine residues are metal ligands in that centre but not in the Cu_B centre. Revealing the exact roles of CtaG and YpmQ in assembly of cytochrome caa_3 in Gram-positive bacteria remains a challenge.

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