ELSEVIER

Biochimica et Biophysica Acta 1232 (1995) 67–74

BBA Biochimica et Biophysica Acta

Functional analysis of subunits III and IV of *Bacillus subtilis* aa_3-600 quinol oxidase by in vitro mutagenesis and gene replacement

Gaetano Villani [a,b], Maria Tattoli [a], Nazzareno Capitanio [a], Philippe Glaser [b], Sergio Papa [a,*], Antoine Danchin [b]

[a] *Institute of Medical Biochemistry and Chemistry, University of Bari, Bari, Italy*
[b] *Unité de Régulation de l 'Expression Génétique, Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris Cedex 15, France*

Received 25 April 1995; accepted 4 August 1995

Abstract

Using the high efficiency of homologous gene recombination in *Bacillus subtilis*, a strategy for mutational analysis of the proton pumping aa_3-600 quinol oxidase of this organism has been developed. The *qox* operon with the *qoxA*, *qoxB*, *qoxC* and *qoxD* genes, coding for the four subunits of this oxidase, was deleted and then replaced with mutated copies in which *qoxC* (subunit III) or *qoxD* (subunit IV) genes were deleted. The complete deletion of the *qox* operon caused disappearence of heme aa_3-600 and a slight depression of the overall respiratory activity, compensated by alternative oxidase with no proton pumping activity. Deletion of *qoxC* probably resulted in a defective assembly of the aa_3-600 quinol oxidase. The strain with deletion of *qoxD* gene expressed normal content of heme aa_3-600 but exhibited a reduced respiratory activity and a significantly depressed proton pumping activity. These results show that subunit IV is critical for the activity of the proton pumping aa_3-600 quinol oxidase.

Keywords: Cytochrome; Cytochrome oxidase; Prokaryotic quinol oxidase; (*B. subtilis*)

1. Introduction

The aa_3-600 quinol oxidase is one of the terminal oxidases of the branched respiratory chain of the aerobic Gram-positive *Bacillus subtilis*. This organism can synthesize *a*, *b*, *c*, *d*, and *o*-type cytochromes (for a review see Ref. [1]) and at least four terminal oxidases: the caa_3-type cytochrome-*c* oxidase, the aa_3-600 and *d*-type quinol (menaquinol) oxidases and *b* and/or *o*-type oxidase [2–4]. The aa_3-600 quinol oxidase is predominantly expressed during vegetative growth. Cytochrome aa_3-600 belongs to the superfamily of the heme-copper oxidases and contains three active redox centres, two hemes (heme *a* and a_3) and one copper (Cu_B). The α-band absorption maximum of the reduced minus oxidised enzyme is shifted by 5–7 nm to the blue, if compared to the majority of the other aa_3-type oxidases. Like other prokaryotic quinol oxidases [5,6], the aa_3-600 misses the second copper centre (Cu_A) [7], which has been demonstrated to be binuclear in *Bacillus subtilis* caa_3 [8].

The aa_3-600 quinol oxidase is apparently a four subunit enzyme encoded by the *qox* operon [9], subunits I, II, III and IV being respectively encoded by the genes *qoxB*, *qoxA*, *qoxC* and *qoxD*.

The catalytic subunit I and subunit III present a high degree of similarity with those of *bo* quinol oxidase of *E. Coli* (*cyo* operon) [10] and caa_3-605 cytochrome-*c* oxidase of *B. subtilis* (*cta* operon) [9,11] (51% with *cyoB* and 48% with *ctaD* for subunit I, and 45% with *cyoC* and 49% with *ctaE* for subunit III). Subunit II, although with a similar hydropathy profile, shows a lower level of amino acid identity (31% with *cyoA* and 25% with *ctaC*). The role of these three subunits, corresponding to the mitochondrial encoded subunits of the eukaryotic cytochrome-*c* oxidase has been largely investigated in prokaryotes. In contrast, very little is known on the function of the poorly conserved fourth subunit of the bacterial oxidases whose sequence is not significantly similar to any nuclear encoded subunit sequence of the eukaryotic enzymes.

As other members of the heme-copper oxidase superfamily, the aa_3-600 quinol oxidase has a vectorial proton translocation activity [12]. Thus, it contributes to the transmembrane electrochemical gradient by a proton pumping

* Corresponding author. Fax: +39 80 5478429.

SSDI 0005-2728(95)00112-3

activity (vectorial protons) and a charge separation deriving from the anisotropy of the aerobic oxidation of menaquinol (scalar protons).

In this study, using the high efficiency of homologous gene recombination in *B. subtilis*, we have set up a strategy for systematic mutational analysis of the aa_3-600 quinol oxidase, based on the use of allelic exchange for the insertion of mutations directly on the bacterial chromosome. This strategy mainly consists of two-step gene replacement of the *qox* operon in the chromosome of *B. subtilis*. The operon is first deleted and then replaced with mutated copies in which *qoxC* (subunit III) and *qoxD* (subunits IV) genes are deleted respectively.

By using this method, here we show for the first time that in *B. subtilis* subunit IV is critical for the expression of a normal proton pumping aa_3-600 quinol oxidase and that there is an alternative oxidase which does not act as a proton pump.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The *Bacillus subtilis* strain 168 was used as wild-type strain. *Escherichia coli* (*E. coli*) strains used for the plasmid construction and DNA sequencing were the following: XL1-blue [13], TG1 [14], TP610 [15], TP611 [16]. Luria-Bertani (LB) medium was used for standard liquid cultures of *B.subtilis* and *E.coli* [17]. The minimal salt medium (MM) for growth of *B. subtilis* was that of Anagnostopulos and Spizizen [18], supplemented with 0.4% glucose as carbon source and tryptophan to a final concentration of 80 mg/l. Streptomycin resistance of the wild-type and mutant strains was tested by growth on sporulation (SP) plates [19] containing streptomycin at concentrations in the range of 10–100 mg/l. The following antibiotics were used when appropriate: ampicillin 100 mg/l, tetracyclin 20 mg/l, spectinomycin 60 mg/l and kanamycin 5 mg/l.

2.2. DNA methods and genetic techniques

Transformation of *E.coli* strains was performed as described by Chung and Miller [20]. *B. subtilis* cells were transformed as described by Anagnostopulos and Spizizen [18]. *B. subtilis* chromosomal DNA was prepared as described by Saunders [21]. Southern blot analysis was performed using a non-radioactive DNA labelling and detection kit from Boehringer Mannheim. Other recombinant techniques were performed as described by Sambrook et al. [22].

2.3. Construction of the mutant strains

Restriction maps of the plasmids constructed throughout this work are shown in Fig. 1. Complete deletion of the *qox* operon was performed using the plasmid pDIA5341 constructed as follows. The *Eco*RV fragment of *B. subtilis* chromosomal DNA containing the *qox* operon with the flanking regions was first excised from Lambda sacPT [9] and cloned at the unique EcoRV site of pBR322, leading to plasmid pDIA5340. The 4.77 kb [kilobase(s)] *Stu*I-*Nco*I fragment was replaced in this last plasmid by the *Stu*I-*Nco*I kanamycin resistant gene from plasmid pDIA5337 [9], giving the final construct pDIA5341. Introduction of modified copies of the *qox* operon was performed using plasmids pDIA5342 and pDIA5343. The first one was obtained by inserting a *Xba*I-*Sma*I filled-in spectinomycin resistance cassette [23] at the unique *Stu*I site of plasmid pDIA5340. The second one was constructed by cloning the *Eco*RV fragment from pDIA5342 in pUC19 digested by *Hin*dIII-*Eco*RI filled-in with Klenow.

Deletion of the *qoxD* gene was obtained by excising a *Nco*I fragment of 296bp [base pair(s)], containing the almost complete gene, from pDIA5342, the resulting construct being named pDIA5345.

Deletion of the *qoxC* gene in pDIA5343 was obtained in two steps. In a first step, a *Sph*I-*Ssp*I fragment of 0.6 kb was subcloned in pUC19 digested by *Sph*I and *Hin*cII. In the second step, the *Rsr*I I-*Eco*RI fragment from pDIA5343 was replaced by the truncated *Rsr*II-*Eco*RI fragment from this intermediate construction, leading to a 0.4 kb *Ssp*I-*Eco*RI deletion in pDIA5343. The final construct was called pDIA5344. The almost complete deletion of the *qoxC* gene was obtained and a new stop codon, 138 bp upstream the initiation codon of the following *qoxD* gene, was created. In this way the translation initiation site of the *qoxD* gene should be unaffected by the mutation.

The strain bringing the complete deletion of the *qox* operon, named Δqox, was obtained by transformation of *B. subtilis* with linearized plasmid pDIA5341 and by selection for kanamycin resistance. B.s.168::SpecR, $\Delta qoxC$ and $\Delta qoxD$ strains were obtained by transformation of the Δqox strain with linearized plasmids pDIA5343, pDIA5344 and pDIA5345 respectively and by selection for spectinomycin resistance (the kanamycin sensitivity being restored). The exact gene replacement was checked in all the mutant strains by Southern blot (data not shown).

2.4. Preparation of solubilized membranes

B. subtilis wild-type and mutant strains were grown in 2-litre flasks at 37°C. For the mutant strains, overnight precultures were made in the presence of the required antibiotics, whereas the large volume cultures were performed without antibiotics to avoid interference in the growth. The bacterial cells were harvested during the exponential phase (turbidity at 600 nm = 0.7–0.9 O.D.) and membranes were essentially prepared as in [4] except that a higher ratio protein/Triton X-100 (0.37 w/w) was used for the solubilization step.

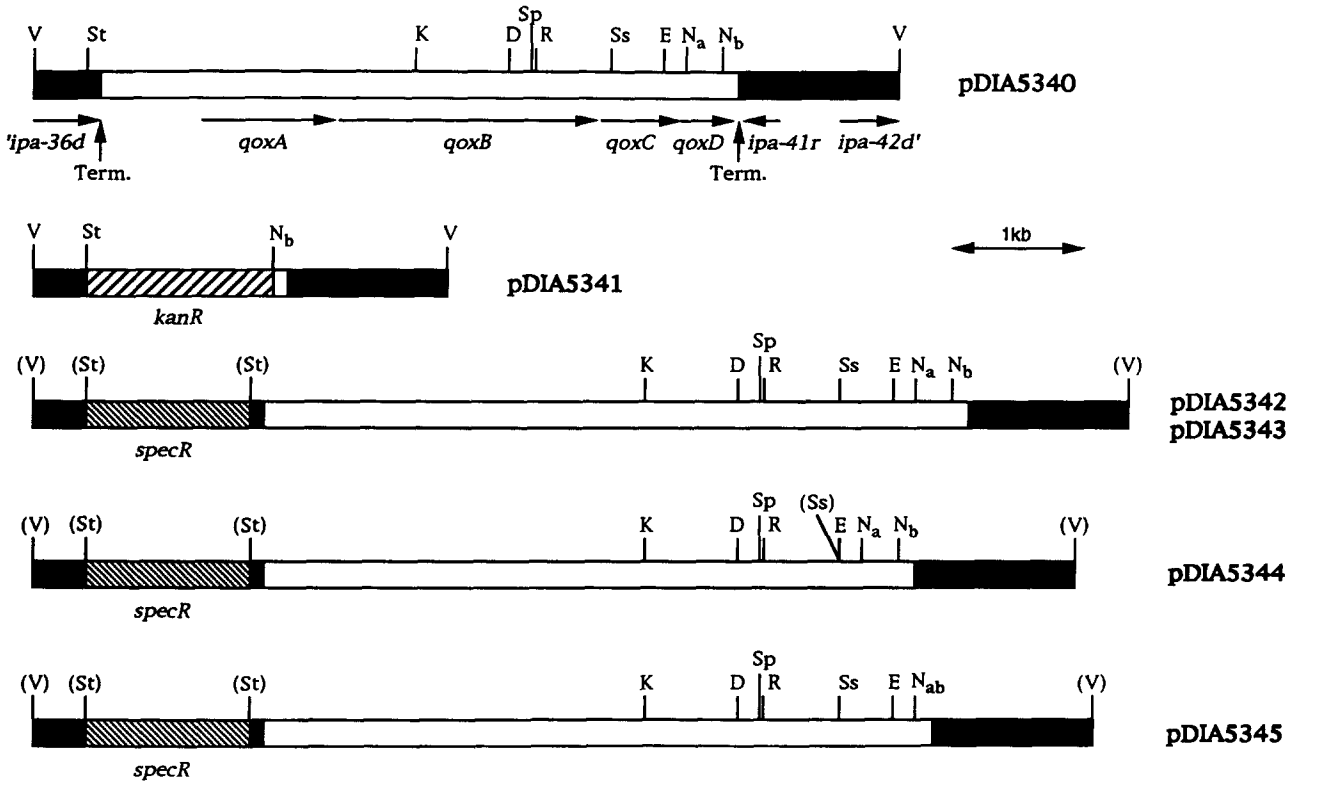

Fig. 1. Restriction map of the inserts of the constructed plasmids. D, *Dra*III; E, *Eco*RI; K, *Kpn*I; N, *Nco*I; R, *Rsr*II; Sp, *Sph*I; Ss, *SSp*I; St, *Stu*I; V, *Eco*RV; *kanR* and *spcR* refer to kanamycin and spectinomycin resistance genes, respectively. Term. indicates transcription termination sequence. Genes are represented by arrows. Lost restriction sites are indicated in brackets. For details see Section 2.

2.5. Optical spectroscopy

Liquid nitrogen temperature spectra were essentially performed as in [9], except that no artificial reductant was added in the sample. In the condition used the sample is assumed to be in anareobiosis with the hemes reduced by endogenous substrates.

In the difference spectra on solubilized membranes, the hemes *c* and *b* contents were estimated, deconvoluting the contributions of both hemes to the absorbance at their specific maxima in the region 550–535 nm and 558–575 nm respectively. The extinction coefficients used were $\Delta\varepsilon_{558-575} = 16.9\ mM^{-1}$, $\Delta\varepsilon_{550-535} = 5.5\ mM^{-1}$ for cytochrome *b* [7] and $\Delta\varepsilon_{558-575} = 2.9\ mM^{-1}$, $\Delta\varepsilon_{550-535} = 23.97\ mM^{-1}$ for cytochrome *c* [24] and the concentrations calculated by the following equations:

$$[\text{cyt. } b] = 0.062 \times A_{558-575} - 0.008 \times \Delta A_{550-535}$$

$$[\text{cyt. } c] = 0.420 \times A_{550-535} - 0.229 \times [\text{cyt. } b]$$

Heme a + a_3 content was measured from ΔA at 600–630 nm eventually corrected for baseline slope using a $\Delta\varepsilon = 13.3\ mM^{-1}$ [7].

2.6. Measurements of redox-linked proton translocation and O_2 consumption

Proton translocation was measured electrometrically by the oxygen pulse method. 50 ml of cell at the log growth phase (turbidity at 600 nm = 0.7–0.9 O.D.) were centrifuged and the pellet resuspended in 0.2 ml of the assay medium. 0.1 ml of this cell suspension were added to 3.1 ml of 50 mM KCl, 100 mM KSCN, 0.5 mM Hepes (pH 7.4), in a stoppered glass vessel equipped with a Clark-type oxygen electrode and a fast responding pH electrode. The suspension was thermostated at 25°C and vigorously stirred. The oxygen uptake by endogenous respiration was monitored until full anaerobiosis was achieved; after 5 min equilibration the pH of the suspension was 6.5–6.7 and proton release was elicited by adding 2.5 μM O_2 as air-equilibrated bidistilled water at 25°C. pH changes were quantitated by titrated 10 mM HCl additions.

For oxygen consumption measurements, the harvested cells were resuspended to and OD_{600} of 10 in LB (Luria-Bertani) medium. 50 μM of this cell suspension were added to 1.6 ml of the same medium and the respiration monitored polarographically. All the assays were repeated several times and results were found to be reproducible within 10%.

For the inhibitor-titration, HOQNO or KCN were added when a stationary respiratory rate was achieved and the inhibited respiratory rate was also measured under stationary conditions (5 min after the addition of the inhibitor); this was critical in particular for KCN inhibition at subsaturating concentrations for some of the mutant strains tested, where a sluggish phase was observed (see text and Fig. 6).

The I_{50} was calculated best fitting the respiratory rates vs the inhibitor concentrations plot by the following empirical equation:

$$V_I/V_O = 1 - [I]^n/([I]^n + I_{50})$$

where: V_I = respiratory rate at a given inhibitor concentration; V_O = uninhibited respiratory rate; [I] = concentration of the inhibitor; n = exponential factor; I_{50} = concentration of the inhibitor giving 50% inhibition.

3. Results

The strategy of mutagenesis is schematically presented in Fig. 2. It consists mainly of a two-step gene replacement method, employing spectinomycin (*specR*) and kanamycin (*kanR*) resistance genes as selective markers. In the first step (deletion step) the complete *qox* operon was removed from the chromosome of *B. subtilis* and replaced by the *kanR* gene. In the second step, a modified copy of the *qox* operon flanked by the specR gene was introduced in the chromosome of the *Δqox* strain, replacing the *kanR* gene. The position of the *specR* gene was chosen in order not to affect the expression of the *qox* operon. However, this marker interrupts the upstream *ipa-36d* gene, presumed to code for a galactose 1-p uridyltransferase [16]. Comparison of the wild type strain and the mutant strain B.s.168::SpecR (carrying only the insertion of the *specR* gene in the chromosome) showed no difference as far as shape of colonies, streptomycin resistance, spectra and other biochemical parameters tested in this work, are concerned. By using this method the mutant strains *Δqox*, *ΔqoxC* and *ΔqoxD*, harbouring deletion of the whole *qox* operon, of the *qoxC* gene and *qoxD* gene respectively, were constructed.

Since the streptomycin resistance has been largely used to isolate strains carrying mutations affecting cytochrome aa_3 in *B. subtilis* [25,26], we tested our mutant strains on plates containing streptomycin (see Table 1). The mutant strain *Δqox* showed in fact a strong resistance to this antibiotic, confirming the previous results on mutants lacking aa_3-type cytochromes [26,27] and, in particular, on the *B. subtilis* strain where the *qox* operon had been interrupted [9]. The streptomycin sensitivity was partially restored in the *ΔqoxC* mutant, whereas no reversion was observed in the strain *ΔqoxD* (resistant up to 100 mg/l of streptomycin).

The low-temperature absolute spectrum of intact cells of the *B. subtilis* 168 wild-type strain shows the α-band absorption maximum at ≈ 600 nm and γ-band shoulder at ≈ 440 nm, characteristic of the reduced cytochrome aa_3 of quinol oxidase in this species, evidently kept reduced by endogenous substrates (Fig. 3). Both the α-peak and γ-shoulder of cytochrome aa_3 were absent in the *Δqox*

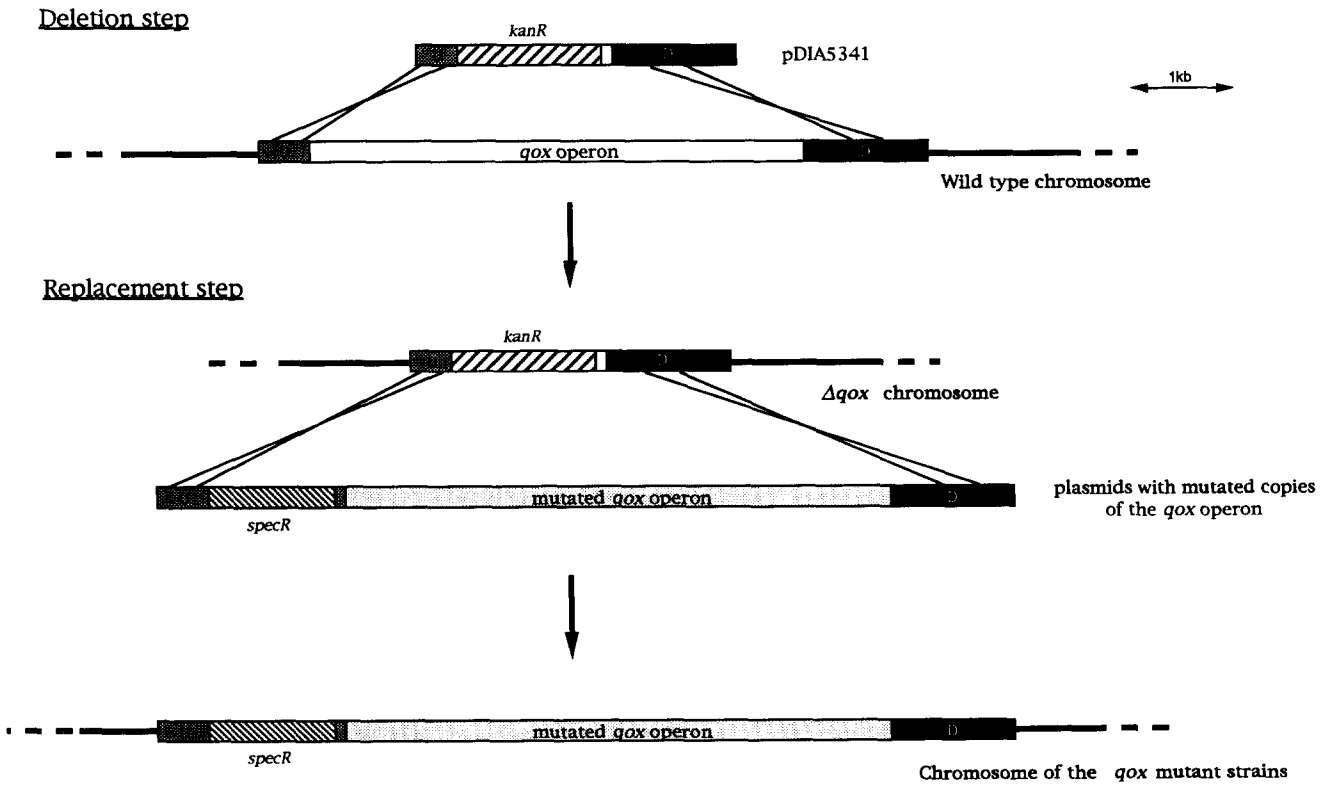

Fig. 2. Strategy of mutagenesis. U and D refer to the upstream and downstream regions of the *qox* operon coding for the aa_3-600 quinol oxidase; *kanR* and *specR* refer to the kanamycin and spectinomycin resistance genes respectively. Crossed lines indicate a single crossing-over event.

Table 1
Phenotypes of *Bacillus subtilis* strains

	B.s. 168	Δqox	ΔqoxC	ΔqoxD
Heme *a* [a]	0.35	n.d.	0.13	0.35
Heme *b* [a]	0.71	0.58	0.62	0.51
Heme *c* [a]	n.d. [f]	0.19	n.d.	0.18
O_2 consumption [b]	182±6	125±7	299±8	88±5
KCN [c]	35	177	395	76
HOQNO [c]	0.07	0.4	1.0	1.0
H^+/e^-	2.0	1.0	0.9	1.2
Power output [d]	364	125	269	106
Strep. res. [e]	–	++	+	+++

Heme contents in the membranes, streptomycin resistance, respiratory activity, H^+/e^- for respiration-linked proton ejection and sensitivity of respiration to inhibition by KCN and HOQNO (for more details see Section 2).
[a] nmol of heme/mg membrane proteins; [b] μM e^-/min/OD_{600}; [c] I_{50} as μM concentration; [d] μM H^+/min/$O.D._{600}$; [e] streptomycin resistance tested in a range of 10–100 mg/l; [f] not detectable.

mutant in which the operon was completely deleted. Deletion of the *qoxC* gene (subunit III) or of the *qoxD* gene (subunit IV) had no detectable effect on the position of the α-peak and γ-shoulder of reduced cytochrome aa_3 of quinol oxidase in intact cells (Fig. 3). This spectroscopic pattern was confirmed by difference (reduced-minus-oxidised) spectra of solubilized membranes showing in the wild type strain, *ΔqoxC* and *ΔqoxD* mutants the difference α-peak at 600 nm of cytochrome aa_3 (Fig. 4). Difference spectra showed also, in the wild-type and mutant strains a large absorption peak at 556–568 nm typical of *b* cytochrome (Figs. 3 and 4). Occurrence in *Bacillus subtilis* cytoplasmic membrane of a cytochrome *b-558* of succinate-menaquinone reductase [27,28] and of different *b*-type cytochromes which bind carbon monoxide and could represent alternative oxidases has been reported [2,3] (see however Ref. [12]). In Fig. 5 CO-reduced minus reduced difference spectra for the wild-type strain and the *Δqox* mutant are presented. The positive contribution of heme *a* type at 430 and 590 nm and the negative contribution at 444 nm visible in the wild-type are absent in the *Δqox*

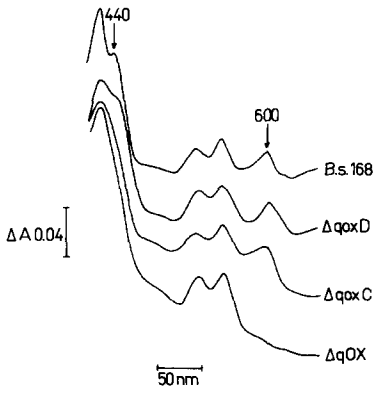

Fig. 3. Absolute spectra of *B. subtilis* strains. Spectra were recorded at liquid nitrogen temperature on whole-cell suspension.

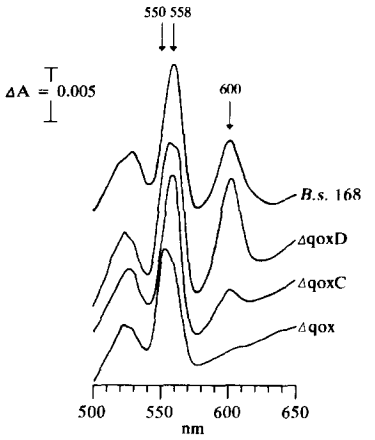

Fig. 4. Difference optical spectra of solubilized membranes from wild-type and mutant strains of *B. subtilis*. Membranes were prepared as described in Section 2 and suspended in Tris [Tris(hydroxymethyl)-methylamine]-HCl 50 mM (pH 8.0), NaCl 150 mM, 3% Triton X-100, at a protein concentration of 1–1.5 mg/ml. The spectra were scanned at room temperature with the reference sample oxidised by 0.1 mM ferricyanide and the measure sample reduced by a few grains of dithionite.

mutant. Positive contribution of heme *b* type at 418 nm and negative contribution at 428 and 558 are clearly visible both in the wild and *Δqox* strains.

Deconvolution of difference spectra revealed the appearance of a detectable amount of cytochrome *c* in the

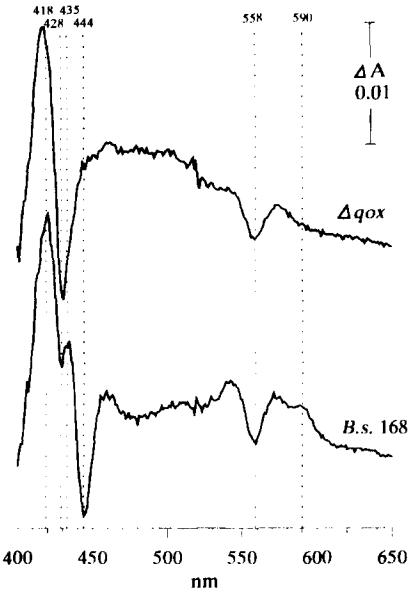

Fig. 5. CO-reduced minus reduced difference spectra of *B. subtilis* membranes. Membranes from wild type and *Δqox* strains were prepared as reported in Section 2 and suspended in the medium described in Fig. 4 to a final concentration of 2 mg prot/ml. The reduced form of the membraneous cytochromes was obtained by addition of a few grains of $Na_2S_2O_4$ and the room-temperature spectra stored in the memory of a diode array spectrophotometer; then CO was bubbled through the suspension, supplemented with a small amount of antifoam, for 2 min in the dark, the CO-reduced spectra taken and the stored reduced spectra subtracted from it. The CO-reduced species were spectrally stable for at least one hour.

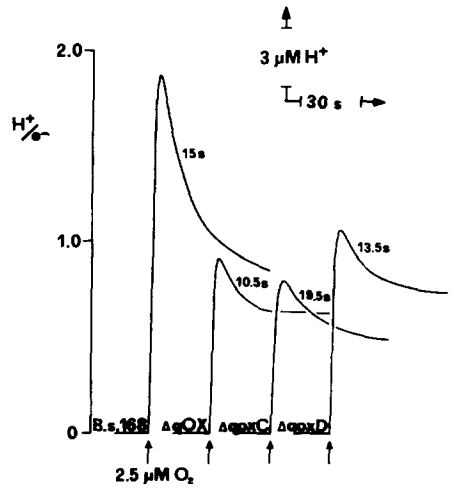

Fig. 6. Redox-linked proton translocation measured in cells of wild-type and mutant strains of *B. subtilis*. For experimental conditions see Section 2. The figures on the curves refer to the $t_{1/2}$ of the proton backflow following the initial release (see text).

strains *Δqox* and *ΔqoxD* (Table 1); this was also confirmed by ascorbate reduced-minus-oxidised spectra on solubilized membranes (data not shown).

It should be noted that, under the exponential growth conditions used, no spectroscopic signal of the *d*-type cytochrome (showing an absorption maximum at about 630 nm) was observed either in the wild-type or in the mutant strains.

The respiratory proton pumping activity of *B. subtilis* strains was examined by oxygen pulses of freshly harvested log-phase cells resuspended in a KSCN-KCl medium made anaerobic by endogenous respiration (Fig. 6). As shown by Lauraeus and Wikström [12], in the wild-type strain of *B. subtilis*, oxygen pulses resulted in proton ejection with an H^+/e^- (ratio of protons translocated per electron transferred to dioxygen) of 2, due to the activity of the aa_3-600 quinol oxidase. *Bacillus subtilis* expresses, apparently, only a NADH-quinone oxidoreductase which does not pump protons [12]. It has to be recalled that oxidation of hydrogenated carriers (menaquinol) by cytochromes takes place at the outer side of the plasma membranes so as to result directly in the scalar release of 1 H^+/e^-. The additional proton release is contributed by vectorial proton pumping from the cytoplasmic to the external medium, this amounting in the wild-type strain to 1 H^+/e^- [12]. The Table summarizes the H^+/e^- ratios expressing the overall proton pumping capacity of the respiratory chains of the *B. subtilis* strains (see also Fig. 6), the respiratory activities, the content of cytochromes and the concentrations of KCN and HOQNO [(2-heptyl-4-hydroxyquinoline), classical inhibitor of quinol oxidases] giving 50% inhibition of respiration.

The complete deletion of the *qox* operon (*Δqox* strain), resulting in disappearance of cytochrome aa_3 (Figs. 3 and 4), exhibited only a small depression of respiratory activity, no significant change in the content of *b* cytochromes, appearance of cytochrome *c*, full suppression of vectorial proton pumping (only scalar release of 1 H^+/e^- was in fact detected in this strain). The depression of proton pumping observed in the *Δqox* and other *B. subtilis* mutants did not result from enhanced rate of passive proton back-flow. Indeed, the $t_{1/2}$ for proton re-entry observed upon anaerobiosis was practically the same in all the mutants as compared to the wild-type strain. The sensitivity to the respiratory inhibitors KCN and HOQNO was significantly decreased.

In the *ΔqoxC* (subunit III deficient) strain, the amount of cytochrome aa_3-600 detected in the solubilized membranes was about one-third of that in the wild-type strain, the content of *b*-type cytochromes was practically unchanged as compared to the wild-type strain and no cytochrome *c* was apparently expressed. The respiratory activity was much larger not only as compared to the *Δqox*, but also to the wild-type strain. No vectorial proton pumping was, however, associated with this respiratory activity which, on the other hand, exhibited a decreased sensitivity to inhibition by KCN and HOQNO also as compared to the *Δqox* strain.

In the *ΔqoxD* (subunit IV-deficient) strain, the membrane presented a normal amount of cytochrome aa_3-600, decreased level of *b* cytochromes as compared to the wild-type strain and a substantial content of cytochrome *c*. The respiratory activity was, however, markedly depressed, even below that of *Δqox* strain and exhibited a decreased sensitivity to inhibition by HOQNO. In the *ΔqoxD* strain the efficiency of respiratory proton pumping was decreased to an H^+/e^- ratio of 1.2.

Another anomalous feature of the respiratory activity of the *Δqox*, *ΔqoxC* and *ΔqoxD* mutant strains was represented by a pronounced lag in inhibition by KCN as compared to the wild-type strain, where respiration was immediately blocked upon addition of the inhibitor (Fig. 7).

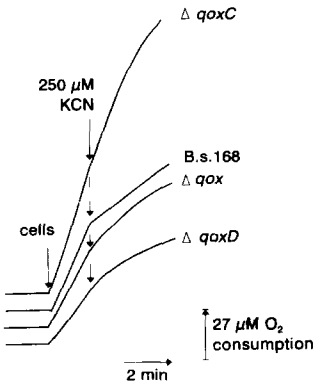

Fig. 7. Oxygen consumption and cyanide inhibition in cells of wild-type and mutant strains of *B. subtilis*. For experimental conditions see Section 2.

4. Discussion

Taking advantage of the homologous gene recombination in *Bacillus subtilis*, we have developed in this work an efficient tool for mutational analysis of the aa_3-600 quinol oxidase. This is based on a two-step gene replacement method and utilizes antibiotic resistance genes as selective markers. It allows to introduce deletions or site-directed mutations in the *qox* operon directly in the chromosome, so representing a less disputable alternative to the usual complementation technique so far applied for the mutational analysis of bacterial cytochrome oxidases [29]. Performing mutagenesis on the chromosomal copy version of a gene, instead of using a plasmid-borne copy, leads to several advantages for structural and physiological analysis: (i) stability of the DNA constructs within the cells; (ii) expression of the mutated operon at physiological levels; (iii) respect of the normal internal stoichiometry of the subunits encoded by the operon. Our strategy is evidently made possible by the fact that the aa_3-600 quinol oxidase is not essential for the growth of *B. subtilis*. This Gram-positive bacterium has in fact a branched respiratory chain with at least four terminal oxidases.

The low temperature absolute spectra of intact cells and differential (reduced-minus-oxidised) spectra of solubilized membranes show that, under the growth conditions used in our experiments (exponential phase in a rich medium), the *B. subtilis* 168 strain expresses the aa_3-600 quinol oxidase, but not the caa_3-605 oxidase, neither the *d*-type oxidase. The absence of any cytochrome-*c* oxidase activity was confirmed by the finding that cyanide-sensitive respiration supported by ascorbate plus TMPD (N,N,N′N′-tetramethyl-*p*-phenylendiamine) was negligible both in the wild and in the mutant strains (data not shown). The wild-type strain seems also to express *b*-type CO-binding cytochromes (see Figs. 3–5); however, the KCN-titration of the respiratory activity of the cells, giving an I_{50} typical of the *a*-type oxidases, indicates that *b*-type cytochromes should be inactive in the wild-type strain.

Spectroscopic and functional characterization of the *B. subtilis* mutant *Δqox* shows that deletion of the *qox* operon results in the expected disappearance of cytochrome aa_3-600 quinol oxidase. The overall respiratory activity of the mutant was, however, compensated by the alternative oxidase of the *b*-type (or *o*-type). The respiratory activity in this mutant had a diminished sensitivity to KCN (this confirming previous results [2,3]) and to HOQNO; it exhibited only scalar H^+ release (1 H^+/e^-) associated to oxidation of endogenous hydrogen carrier (menaquinol) with no outward proton pumping. Thus the putative *b*-type oxidase of *B. subtilis*, at least in the *Δqox*, as well as in the *ΔqoxC* and *ΔqoxD* mutants, where it might be activated, does not pump protons.

It is worth noting that the overall pumping activity of the *B. subtilis* strains, expressed as the product of the respiratory activity and the H^+/e^- ratio, is inversely related to the streptomycin resistance of the strains (see Table 1). Thus the *Δqox* and *ΔqoxD* strains, which exhibit the lowest power output for proton pumping, are those showing higher resistance to this antibiotic. It might be noted that these strains also show the same phenotype as far as shape of colonies (smaller than the wild-type) and content of cytochrome *c* are concerned.

Phenotypic characterization of the *B. subtilis* mutant obtained by replacement of the *qox* operon with the mutated copy of the operon-harbouring specific deletion of the *qoxC* gene may indicate that subunit III, encoded by this gene, is essential for the proper assembly of a functional, proton pumping aa_3-600 quinol oxidase. The reduced content of the cytochrome aa_3-600 in the membrane could result from a defective association with the membrane of subunit I and maybe of subunits II and IV. Evidence that deletion of subunit III leads to defective assembly of cytochrome-*c* oxidase in *Paracoccus denitrificans* has also been reported [30,31]. The *ΔqoxC* mutant of *B. subtilis* exhibits, in fact, a respiratory activity which is even larger than that of the wild-type strain. Since no expression of cytochrome *d* and caa_3-605 could be detected even in this mutant, it seems possible that the higher respiratory rate, which has a diminished sensitivity to KCN and HOQNO, even as compared to the *Δqox* strain, and does not result in proton pumping (as revealed by an overall proton translocation of 1 H^+/e^-), represents an increased activity of alternative oxidase and/or an anomalous activity of improperly membrane-assembled subunits I, II and may be IV of the aa_3-600 oxidase. However, this residual activity of the subunit III-depleted enzyme should still maintain anisotropy of menaquinol oxidation, as indicated by the release of 1 H^+/e^-.

Deletion of the qoxD gene (subunit IV) did not affect the content of cytochrome aa_3-600 in the membrane. The respiratory activity of this mutant was, however, impaired and its sensitivity to HOQNO, and to some extent also to KCN, was decreased. Proton pumping in the *ΔqoxD* strain was largely suppressed, as indicated by the lower stoicheiometry of the total proton ejection (1.2 H^+/e^-). This could be due to greater contribution to the overall respiratory activity of the alternative *b*-type oxidase and/or decoupling of proton pump in the defective enzyme. In any event the observations show that subunit IV is essential for the functional activity of the proton pumping aa_3-600 quinol oxidase of *Bacillus subtilis*.

Acknowledgements

We are grateful to Dr. Pierre Labbe for help in the low-temperature spectrophotometric experiments. We express our gratitude to Prof. Carlo Di Benedetta, Dr. Francesco Vitiello and Dr. Gianfranco Gennarini of the Institute of Human Physiology, University of Bari, for having kindly made available their laboratory facilities.

We thank Dr. Frank Kunst, Dr. Marie Francoise Hullo, Ivan Moszer and Margarida Santana for helpful discussion. We would also like to thank Dr. Octavian Barzu for critical reading of this manuscript.

References

[1] von Wachenfeldt, C. and Hederstedt, L. (1992) FEMS Microbiol. Lett. 100, 91–100.
[2] De Vrij, W., Van den Burg, B. and Konings, W., N. (1987) Eur. J. Biochem. 166, 589–595.
[3] James, W.S., Gibson, F., Taroni, P. and Poole, R.K. (1989) FEMS Microbiol. Lett. 58, 277–282.
[4] De Gier, J.-W.,L., Lubben, M., Reijnders, W.N.M., Tipker, C.A., Slotboom, W.N.M., Van Spanning, R.J.M., Stouthamer, A.H. and Van der Oost, J. (1994) Mol. Microbiol. 13, 183–196.
[5] Puustinen, A., Finel, M., Haltia, T., Gennis, R.B. and Wikstrom, M. (1991) Biochemistry 30, 3936–3942.
[6] Annemuller, S. and Schafer, G. (1990) Eur. J. Biochem. 191, 297–305.
[7] Lauraeus, M., Haltia, T., Saraste, M. and Wikstrom, M. (1991) Eur. J. Biochem. 197, 699–705.
[8] von Wachenfeldt, C., De Vries, S. and Van der Oost, J. (1994) FEBS Lett. 340, 109–113.
[9] Santana, M., Kunst, F., Hullo, M.F., Rapoport, G., Danchin, A. and Glaser, P. (1992) J. Biol. Chem. 267, 10225–10231.
[10] Chepuri, V., Lemieux, L., Au, D.C.-I. and Gennis, R.B. (1990) J. Biol. Chem. 265, 11185–11192.
[11] Saraste, M., Metso, T., Nakari, T., Jalli, T., Laureaus, M. and Van der Oost, J. (1991) Eur. J. Biochem. 195, 517–525.
[12] Lauraeus, M. and Wikstrom, M. (1993) J. Biol. Chem. 268, 11470–11473.
[13] Bullock, W.O., Fernandez, J.M. and Short, J.M. (1987) Bio-Techniques 5, 376.
[14] Gibson, T.J. (1984) Ph.D. thesis, University of Cambridge, Cambridge, UK.
[15] Hedegaard, L. and Danchin, A. (1985) Mol. Gen. Genet. 201, 38–42.
[16] Glaser, P., Kunst, F., Arnaud, M., Coudart, M.P., Gonzales, W., Hullo, M.F., Ionescu, M., Lubochinsky, B., Marcelino, L., Moszer, I., Presecan, E., Santana, M., Schneider, E., Schweizer, J., Vertes, A., Rapoport, G. and Danchin, A. (1993) Mol. Microbiol. 10, 371–384.
[17] Miller, J.H. (1972) in Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
[18] Anagnostopoulos, C. and Spizizen, J. (1961) J. Bacteriol. 81, 741–746.
[19] Schaeffer, P., Millet, J. and Aubert, J.P. (1965) Proc. Natl. Acad. Sci. USA 54, 704–711.
[20] Chung, C.T. and Miller, R.H. (1988) Nucleic Acids Res. 16, 3580.
[21] Saunders, C.W., Schmidt, B.J., Morot, M.S., Thompson, L.D. and Guyer, M.S. (1984) J.Bacteriol. 157, 718–726.
[22] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) in Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
[23] Murphy, E. (1985) Mol. Gen. Genet. 200, 33–39.
[24] Berry, E.A. and Trumpower, L. (1987) Anal. Biochem. 161, 1–15.
[25] Arrow, A.S. and Taber, H.W. (1986) Antimicrob. Agents Chemother. 29, 141–146.
[26] Taber, H.W., Mueller, J.P., Miller, P.F. and Arrow, A.S. (1987) Microbiol. Rev. 51, 439–457.
[27] Hagerall, C., Aasa, R., von Wachenfeldt, C. and Hederstedt, L. (1992) Biochemistry 31, 7411–7421.
[28] Magnusson, K., Philips, M.K., Guest, J.R. and Rutberg, L. (1986) J. Bacteriol. 162, 1067–1071.
[29] Hosler, J.P., Ferguson-Miller, S., Calhoun, M.W., Thomas, J.W., Hill, J., Lemieux, L.J., Ma, J., Georgiou, C., Fetter, J., Shapleigh, J., Tecklenburg, M.M.J., Babcock, G.T. and Gennis, R.B. (1993) J. Bioenerg. Biomembr., 25, 121–136.
[30] Haltia, T., Finel, M., Harms, N., Nakari, T., Raitio, M., Wikstrom, M. and Saraste, M. (1989) EMBO J. 8, 3571–3579.
[31] Haltia, T., Saraste, M., Wikstrom, M. (1991) EMBO J. 10, 2015–2021.