Targeted gene-replacement mutagenesis of *dcrA*, encoding an oxygen sensor of the sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough

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A gene-replacement mutagenesis method has been developed for the anaerobic, sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough and used to delete *dcrA*, encoding a potential oxygen or redox sensor with homology to the methyl-accepting chemotaxis proteins. A suicide plasmid, containing a cat-marked *dcrA* allele and a counter-selectable *sacB* marker was transferred from *Escherichia coli* S17-1 to *D. vulgaris* by conjugation. Following plasmid integration the desired *dcrA* deletion mutant (*D. vulgaris* F100) was obtained in media containing sucrose and chloramphenicol. Southern blot screening was required to distinguish *D. vulgaris* F100 from strains in which the *sacB* marker was inactivated by transposition of an endogenous IS element. No anaerotactic deficiency has so far been detected in *D. vulgaris* F100, which was found to be more resistant to inactivation by oxygen than the wild-type. Increased transcription of the *rbo-rub* operon, located immediately downstream from *dcrA*, was demonstrated by Northern blotting and may be the cause of this unusual phenotype, in view of the recent discovery that Rbo can complement the deleterious effects of superoxide dismutase deficiency in *E. coli*.

Keywords: gene replacement, oxygen sensor, aerotaxis, superoxide dismutase, *Desulfovibrio*

INTRODUCTION

Anaerobic, sulfate-reducing bacteria such as those of the genus *Desulfovibrio* are gradient organisms that position themselves in niches with optimal chemical parameters in soils and aquatic sediments. In sediments these parameters (e.g. the concentrations of oxygen and sulfide) vary over modest distances (0 to 20 cm) in the direction perpendicular to the water–sediment interface (Lillebaek, 1995). Steeper gradients and, as a consequence, narrower distributions of bacteria were demonstrated in a 4 mm thick, photosynthetic biofilm, in which sulfate-reducing bacteria were present. The zone occupied by the bacteria shifted towards the base when surface oxygen concentrations increased during illumination (Ramsing *et al.*, 1993), indicating bacterial

motility in response to light-induced changes in the oxygen concentration gradient.

We have shown that *D. vulgaris* Hildenborough has at least one chemoreceptor (DcrA) that serves as a sensor of the oxygen concentration or redox potential of its surroundings (Fu *et al.*, 1994). Although its function in chemotaxis remains to be demonstrated, DcrA has the structural design of the methyl-accepting chemotaxis proteins (MCPs) found in the *Enterobacteriaceae*, with an N-terminal, periplasmic, sensing domain and a C-terminal, cytoplasmic, signalling domain. The sensing domain contains a c-type haem and the degree of methyl-labelling of the signalling domain was found to be influenced by addition of oxygen (decreased labelling) and of the reductant dithionite (increased labelling). Thus DcrA and the 15 other members of the *D. vulgaris* chemoreceptor family (Deckers & Voordouw, 1994) may help the organism to find its optimal ecological niche.

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Elucidation of the function of DcrA requires a genetic approach in which the phenotypic change of a targeted gene replacement mutant is assessed relative to the wild-type. Unfortunately, the genetics of the sulfate-reducing bacteria have lagged far behind their physiological and biochemical characterization (Wall, 1993). Conjugal transfer of broad-host-range plasmids belonging to incompatibility group IncQ is presently the only gene transfer method available for *D. vulgaris* (Argyle *et al.*, 1992; Powell *et al.*, 1989; van den Berg *et al.*, 1989). Attempts to use conjugation of suicide plasmids for construction of specific gene mutants of *D. vulgaris* were unsuccessful (van den Berg *et al.*, 1989; van Dongen *et al.*, 1994). The intriguing biochemical properties of DcrA provided a strong incentive to revisit the problem of gene interruption or replacement in *D. vulgaris*. We report here the replacement of *dcrA* with a *cat* gene marker and describe the phenotype effects of this replacement.

METHODS

Bacterial strains, plasmids and growth conditions. Bacteria and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37 °C in tryptone/yeast extract (TY) medium or on TY agar plates with antibiotics as

required (Voordouw *et al.*, 1989). Liquid cultures of *D. vulgaris* were grown in medium C (Postgate, 1984) at 35 °C in an anaerobic hood (Forma Scientific) containing a gas mixture of 5% (v/v) H₂, 10% (v/v) CO₂ and 85% (v/v) N₂. Strains used for chemotactic studies were first grown on a medium C swarm plate (0.1%, w/v, agar) to select motile cells and then grown in liquid medium C or in TY medium overnight before use. Plating and colony purification of *D. vulgaris* was done by spreading diluted liquid cultures (containing approx. 50 c.f.u.) on medium E plates (Postgate, 1984), containing appropriate antibiotics, at 35 °C in the same gas atmosphere. *D. vulgaris* strains were stored as liquid cultures in medium B (Postgate, 1984) at 4 °C.

Mating of *D. vulgaris* with *E. coli* S17-1 was done on a medium E plate supplemented with 1.5 g KNO₃ l⁻¹ to allow growth of *E. coli* in the anaerobic hood (Powell *et al.*, 1989). Integrant selection was on a medium E plate containing chloramphenicol (Cm; 10 µg ml⁻¹) and kanamycin (Km; 50 µg ml⁻¹). These concentrations of antibiotics were used throughout in both liquid cultures and plating media unless otherwise stated. Medium C or medium E containing Cm and sucrose (2.5 to 10%, w/v) were used for enrichment and selection of gene replacement mutants.

Integration plasmid construction. Plasmid pDcr1 was cut with *Hind*III and *Bam*HI, and religated after end-filling to remove these sites (Fig. 1). The resulting plasmid was cut with *Pst*I and *Mlu*I, and ligated with a *Bam*HI linker after end-

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype and/or phenotype	Reference
<i>Desulfovibrio vulgaris</i>		
Hildenborough	NCIMB 8303; wild-type, Km ^R Cm ^S Suc ^R	Postgate (1984)
F1	With pΔDcrA2CTB integrated into the chromosome; Km ^R Cm ^R Suc ^R	This study
F1SR	All F1 derivatives with <i>sacB</i> gene mutated; Km ^R Cm ^R Suc ^R	This study
F100	<i>dcrA</i> gene replaced with a <i>cat</i> gene cassette from pUC19Cm; Km ^R Cm ^R Suc ^R	This study
<i>Escherichia coli</i>		
TG2	Δ(<i>lac-pro</i>) <i>supE thi hsdM hsdR recA</i> F' (<i>traD36 proAB⁺ lacZΔM15I^q</i>)	Sambrook <i>et al.</i> (1989)
S17-1	<i>thi pro hsdR recA</i> with RP4-2[Tc::Mu,Km::Tn7] in the chromosome; mobilizer strain	Simon <i>et al.</i> (1983)
Plasmid		
pDcr1	pUC8 containing the <i>dcrA</i> gene on a 3.4 kb <i>Ava</i> I- <i>Eco</i> RI fragment; Ap ^R	Dolla <i>et al.</i> (1992)
pJK9Ava1	pUC8 containing the 5' end of the <i>dcrA</i> gene on a 1.4 kb <i>Ava</i> I- <i>Eco</i> RI fragment; Ap ^R	Dolla <i>et al.</i> (1992)
pMOB2	Containing an <i>oriT-sacBR</i> cassette on a 4.8 kb <i>Not</i> I fragment; Km ^R	Schweizer (1992)
pNOT19	pUC19 with 10 bp <i>Nde</i> I- <i>Not</i> I adaptor in <i>Nde</i> I site; Ap ^R	Schweizer (1992)
pSUP104	Broad-host-range vector; Cm ^R	Priefer <i>et al.</i> (1985)
pUC19	Cloning vector, pMB1 origin of replication; Ap ^R	Yanisch-Perron <i>et al.</i> (1985)
pUC19Cm	pUC19 containing in its <i>Bam</i> HI site a 1.4 kb <i>Sac</i> II- <i>Tth</i> III fragment from pSUP104, carrying the <i>cat</i> gene; Ap ^R Cm ^R	This study
pΔDcrA2	A derivative of pDcr1, containing the flanking regions of the <i>dcrA</i> gene of which the <i>Pst</i> I- <i>Mlu</i> I fragment has been replaced with a <i>Bam</i> HI linker; Ap ^R	This study
pΔDcrA2Cm	pΔDcrA2 with a <i>cat</i> gene inserted at the <i>Bam</i> HI site; Ap ^R , Cm ^R	This study
pΔDcrA2N	pΔDcrA2 with the <i>Nde</i> I site changed to <i>Not</i> I; Ap ^R	This study
pΔDcrA2CmN	pΔDcrA2N with a <i>cat</i> gene inserted at the <i>Bam</i> HI site; Ap ^R , Cm ^R	This study
pΔDcrA2CTB	A mobilizable derivative of pΔDcrA2CmN containing an <i>oriT-sacBR</i> cassette from pMOB2 in the <i>Not</i> I site; Ap ^R Cm ^R Suc ^S	This study

Fig. 1. Construction of integration plasmids pΔDcrA2Cm and pΔDcrA2CTB. The thin line indicates DNA from pUC vector; *bla*, gene for β -lactamase; *ori*, pMB1 origin of replication (functional in *E. coli* only). The hatched boxes indicate DNA of the target gene region of *D. vulgaris*: 'up' and 'down', DNA upstream and downstream from the *dcrA* gene. The *cat* gene from pUC19Cm is indicated, as well as the *NotI* cartridge from pMOB2 containing the origin for conjugal transfer (*oriT*) and the counter-selectable gene *sacB* from *B. subtilis*. Relevant restriction sites are: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; M, *Mlu*I; Nd, *Nde*I; No, *Not*I; P, *Pst*I; Pv, *Pvu*II; X, *Xba*I.

repair to form pΔDcrA2 (Fig. 1). A 1.4 kb *Bam*HI fragment from pUC19Cm was then ligated to the *Bam*HI site of pΔDcrA2 to form pΔDcrA2Cm. This plasmid was used directly, or after linearization with *Nde*I, for transformation of *D. vulgaris* by electroporation. For construction of the integration plasmid pΔDcrA2CTB, pΔDcrA2 was cut with *Pvu*II, and the 2.4 kb vector fragment replaced with the equivalent *Pvu*II fragment of pNOT19 to generate pΔDcrA2N. The *Bam*HI fragment from pUC19Cm was next inserted to form pΔDcrA2CmN, followed by insertion of the 4.8 kb *Not*I cassette to give pΔDcrA2CTB (Fig. 1). After confirmation that the inserted *sacB* was functional in an *E. coli* strain containing this plasmid, it was used for conjugal transfer from *E. coli* S17-1 to *D. vulgaris*.

Transformation of *D. vulgaris* by electroporation. Plasmids pΔDcrA2Cm, pSUP104 prepared from *E. coli*, and pSUP104 prepared from *D. vulgaris* were used for transformation of *D. vulgaris* by electroporation as described by Rousset *et al.* (1991). A 100 ml culture of *D. vulgaris*, grown in medium C to OD₆₀₀ 0.4, was suspended in a final volume of 200 μ l. A 35 μ l aliquot of this cell preparation and 3 μ l DNA (1–2 μ g) were transferred into an electroporation cuvette (Bio-Rad), and subjected to electric pulses (2.5 kV, 25 mF and 400–1000 Ω) in a Gene Pulser (Bio-Rad); 1 ml SOC buffer (Dower *et al.*, 1988) was added to the cuvette immediately and the cell mixtures were allowed to recover at 35 °C for 1 h. Aliquots of 200 μ l were then plated onto medium E plates containing Cm. The

plates were incubated for 3 to 7 d. TE buffer (10 mM Tris/HCl, pH 7.4, 1 mM EDTA) replaced the DNA sample in negative controls.

Conjugal transfer and selection for plasmid integration.

E. coli mobilizer strain S17-1 transformants were used as the donor for conjugal transfer of the integration plasmid pΔDcrA2CTB (Fig. 2, pΔ) into recipient *D. vulgaris* using a filter mating method adapted from Powell *et al.* (1989). Plasmid pSUP104 was similarly transferred to determine the conjugation efficiency. Overnight cultures of *D. vulgaris* in medium C (1 ml) and *E. coli* donor cells (0.2 ml) were mixed in a 1.5 ml microfuge tube under anaerobic conditions. After centrifugation the cell mixture was resuspended in 20 μ l medium C, and spread onto a membrane filter (Millipore-MF, 0.22 μ m; diameter 25 mm) placed on a medium E/nitrate mating plate. Following anaerobic incubation for 1 d the filter was transferred to a microfuge tube and the cells resuspended in 1 ml medium C. Aliquots (10 μ l, approx. 10^7 *D. vulgaris* cells, for transfer of pΔDcrA2CTB; 20 μ l 10^{-4} dilution for transfer of pSUP104) were plated onto medium E plates containing Km and Cm. These were incubated for 5 to 7 d and the Cm^r colonies that emerged were restreaked and analysed by Southern hybridization to verify and map plasmid integration.

Sucrose sensitivity assays. Aliquots (1 ml) of fresh, saturated cultures of colony-purified integrants were inoculated

Fig. 2. Strategy used for gene-replacement mutagenesis of *dcrA* in *D. vulgaris* Hildenborough through conjugation. Maps of the suicide integration plasmid pΔDcrA2CTB (pΔ), and the *dcrA* target gene region in *D. vulgaris* wild-type (wt), the plasmid integrant *D. vulgaris* F1 (F1), and the desired gene-replacing second recombination product *D. vulgaris* F100 (F100) are shown with symbols for genes the same as in Fig. 1. The same restriction sites are indicated as in Fig. 1, as well as A, *AccI* and Av, *AvaI*. The DNA regions used as probes in Figs 3, 5 and 6 (thick lines beneath the wt and F1 map), and the sizes of the relevant *AccI* fragments, are also indicated. The homologous recombinations (×) are indicated as: ①, plasmid integrative recombination; ②, gene-replacing, second recombination; ③, reversing, abortive recombination.

anaerobically into screw-capped glass tubes (external diameter 13 mm) containing 5 ml medium C with Cm. Two tubes were inoculated for each culture and growth was monitored with a Klett meter. After 3 h, 0.67 ml 50% (w/v) sucrose was added anaerobically into one of the two tubes. The cell density of both tubes was then monitored regularly for 2 d.

Selection and enrichment of deletion mutants. Liquid enrichment of Cm^R Suc^R cells was done by inoculating 5 to 10 µl of the integrant culture into 5 ml medium C with Cm and sucrose (2.5–10%, w/v) and growing anaerobically for 7 to 9 d until saturation. Chromosomal DNA was prepared from these cultures and analysed by Southern blotting. Cultures containing the desired *dcrA* deletion were next subjected to colony purification on medium E plates with Cm, and DNAs obtained from liquid cultures of isolated colonies were again screened by Southern blot analysis.

Southern blot analysis. Chromosomal DNA of *D. vulgaris* was isolated by a mini-preparation protocol adapted from Marmur (1961), using 1.5 to 5 ml cultures. For verification of plasmid integration, undigested chromosomal DNA was run on an agarose gel and blotted onto a Hybond-N nylon membrane (Amersham). The blot was probed with ³²P-labelled *cat* gene DNA, obtained as a 1.4 kb fragment from plasmid pUC19Cm. This targets the region indicated for the integrant strain F1 in Fig. 2 (F1, *cat*). For the integration mapping and identification of the gene-replacing second recombination, chromosomal DNA was digested to completion with *AccI* or *EcoRI* before agarose gel electrophoresis

and blotting. The blots were analysed by hybridization with ³²P-labelled DNA probes of the *dcrA* gene flanking regions: the *AvaI*–*KpnI* region upstream from the *dcrA* gene and the *MluI*–*EcoRI* region downstream from the *dcrA* gene (Fig. 2, wt, 'up' and 'down'), obtained as 0.58 kb *Bam*HI–*KpnI* and 0.9 kb *Bam*HI–*EcoRI* fragments from plasmids pJK9Aval and pΔDcrA2, respectively. The blots were then stripped of these probes, and re-probed with ³²P-labelled *dcrA* gene (Fig. 2, wt, *dcrA*), obtained as a 2.0 kb *KpnI*–*MluI* fragment from plasmid pDcr1, to confirm the absence of *dcrA*. In addition, a ³²P-labelled *sacB* gene (Fig. 2, F1, *sacB*), obtained as a 2.4 kb *XbaI* fragment from pMOB2, was used as a probe for analysing deletion and insertion mutations of the *sacB* gene. All the above Southern hybridizations were performed under highly stringent conditions as described elsewhere (Sambrook *et al.*, 1989).

Growth properties and stability of the *D. vulgaris* F100 strain.

The Δ*dcrA* strain, *D. vulgaris* F100, and the wild-type were grown separately in medium C in screw-capped tubes and their growth curves were determined by measuring the change in cell density with time using a Klett meter. For assessment of competitiveness under anaerobic conditions the F100 strain was co-cultured with the wild-type in medium C without Cm. Following mixing, a 5% inoculum was used and allowed to grow for 1–2 d before the next transfer. Chromosomal DNAs were prepared from a portion of the initial mixture of the two strains and from each of the subsequent co-cultures. The chromosomal DNAs were restricted with *EcoRI*, and the

digests were analysed by agarose gel electrophoresis and Southern blot hybridization with the downstream region probe to assess changes in the ratio of F100 to wild-type DNA.

For assaying the differential stability under aerobic conditions, fresh cultures of F100 and wild-type were mixed at a ratio of 1:1 or 1:2 (v/v), and 1.5 ml of these mixtures was diluted immediately with 50 ml medium C, which was prepared aerobically and contained resazurin (0.5 mg l^{-1}) as redox indicator. The diluted cell mixtures were then incubated aerobically in 500 ml Erlenmeyer flasks at 37°C with vigorous shaking for up to 6 h. Aliquots of 1.5 ml were taken at different times and moved immediately into the anaerobic hood. To each of these samples, 30 to $60 \mu\text{l}$ $115 \text{ mM Na}_2\text{S}_2\text{O}_4$ solution was added until the light blue colour disappeared. The samples were then re-grown anaerobically at 35°C to saturation. Chromosomal DNAs were prepared from each of the re-grown samples to determine the ratio of the two strains by Southern blot analysis.

Determination of the anaerotactic phenotype. The anaerotactic behaviour of *D. vulgaris* F100 and wild-type was assessed by microchamber and capillary tube assays. The microchamber was made of a glass microscope slide and a coverslip ($60 \times 25 \text{ mm}$, 0.17 mm thick) separated with two narrow strips of a coverslip as spacers between them and sealed with nail-polishing solution along the longer axis to form a rectangular flattened chamber with two open ends. Motile cell samples in medium C or TY medium were transferred out of the anaerobic hood in capped microfuge tubes before use. About $100 \mu\text{l}$ of the anaerobic preparation of motile cells was transferred into the microchamber with a pipette to form a continuous liquid phase along the chamber with a liquid–air interface at both ends. The aerotactic behaviour of motile cells near the liquid–air interface was then examined under a Nikon Labophot light microscope with dark-field illumination.

The capillary tube assay was used to examine the effect of changing the gas phase on cell migration near the medium–gas interface. Micro-measuring capillary tubes ($20 \mu\text{l}$ capacity, 100 mm long; Fisher, catalogue no. 21-164-2D) were autoclaved before use. Two capillary tubes were filled anaerobically with a 5 cm long column of a motile cell preparation of *D. vulgaris* wild-type or F100 by the capillary effect and sealed at the medium phase end with Plasticine (Canada Games Company). Both tubes were then moved out of the anaerobic hood and exposed to air to allow anaerotactic banding of the cells. The tubes were then lined up and loosely connected to a rubber tubing leading to the gas cylinders (either nitrogen or oxygen; Fig. 7a). A gas humidifier containing water was added to the gas line to prevent sample evaporation. Anaerotactic behaviour was examined either visually or with the Nikon Labophot light microscope, which had a narrow slit light stop inserted into the light path to alter the illumination for better visualization of bacteria in the round-shaped capillary tubes.

Northern blot analysis. The effect of deletion of the *dcrA* gene on transcription of the *rbo-rub* operon was investigated by Northern blot assays. *D. vulgaris* wild-type and F100 were grown in 12 ml medium C overnight. Aliquots of 2 ml of these cultures were transferred to 12 culture tubes, which were moved out of the anaerobic hood. One sample of each strain was used immediately for total RNA preparation. The others were exposed to oxygen by shaking in air at 300 r.p.m. Total RNA was extracted from each sample with a rapid isolation protocol (Reddy & Gilman, 1990). The total RNA preparations were analysed by Northern hybridization

(Brown, 1990). Following electrophoresis the gel was stained with ethidium bromide and photographed to ascertain equality of RNA loading from the intensities of the bands for 23S, 16S and 5S rRNA. The RNA was then transferred to a Hybond-N membrane and hybridized with the ^{32}P -labelled insert of plasmid pRbo1 (Brumlik & Voordouw, 1989). Following washing the blot was exposed to the phosphor-imaging plate of a Fuji BAS1000 BioImaging Analyzer for quantification of *rbo-rub* mRNA.

RESULTS

Electroporation of *D. vulgaris*

Electroporation of *D. vulgaris* with circular or linearized pADcrA2Cm failed to give Cm^R transformants. Electroporation of the broad-host-range vector pSUP104 isolated from *E. coli* also failed to give Cm^R transformants, although they could be readily obtained by conjugation (van den Berg *et al.*, 1989; this work, Table 2). The results indicated either that *D. vulgaris* is not readily transformed by electroporation or that a restriction barrier prevents the establishment of foreign DNA. In order to distinguish between these two possibilities, we isolated native pSUP104 from a *D. vulgaris* transconjugant. Electroporation of this native pSUP104 gave approximately 10^3 transformants per μg DNA, while no transformants were obtained upon electroporation of *E. coli* pSUP104 under the same conditions. This result suggested that a restriction/methylation system in *D. vulgaris* prevents the establishment of foreign DNA transferred by electroporation. We therefore resorted to the conjugal transfer approach in which the DNA being transferred is not as susceptible to host restriction (Schweizer, 1992).

Conjugal transfer and integration of a suicide plasmid

Plasmid pADcrA2CTB was transferred by conjugation from *E. coli* S17-1 to *D. vulgaris*. A positive control [mating with *E. coli* S17-1(pSUP104)], and a negative control (mating with nontransformed *E. coli* S17-1) were also done. Following spreading of the mating mixtures onto medium E plates containing Km and Cm, pSUP104 transconjugants appeared after 3 d while pADcrA2CTB integrants (*D. vulgaris* F1) were obtained at a frequency of 1.1×10^{-7} per recipient after 4–5 d anaerobic growth (Table 2). No colonies appeared in plates spread with the negative control mating mixture. Integration of the suicide plasmid was verified by hybridization of the *cat* gene probe to the undigested chromosomal DNA (data not shown). Chromosomal DNAs from two of the integrants were further analysed. Digestion with *AccI* and hybridization with the flanking region probes gave 0.9 and 1.1 kb fragments for *D. vulgaris* wild-type (Fig. 3, lane 2). In the two integrants the 1.1 kb fragment was missing and three new hybridization bands appeared (Fig. 3, lane 3: 1.3 , 2.2 and 4.5 kb), indicating that these two integrants were identical, with integration of pADcrA2CTB through the downstream region of *dcrA* as illustrated in Fig. 2(ⓐ).

Table 2. Results of conjugal transfer and selection of integrants

Plasmid for transfer	Growth time* (d)	Apparent conjugal transfer frequency (no. of transconjugants per recipient cell)	Apparent integration frequency (no. of integrants per recipient cell)	Integration frequency† (no. of integrants per transconjugant cell)
pSUP104	3	$2.3 \times 10^{-2} \pm$	NA	NA
pΔDcrA2CTB	4–5	ND	$1.1 \times 10^{-7} \pm$	4.7×10^{-6}

ND, Not determined; NA, not applicable.

* Time required for appearance of Cm^R colonies on plates containing 10 µg Cm ml⁻¹.

† Estimated by dividing the number of integrants per recipient cell of pΔDcrA2CTB by the number of transconjugants per recipient cell of pSUP104.

± Result is the mean of four experiments.

Fig. 3. Southern blot analysis of integration plasmid pΔDcrA2CTB (lane 1), genomic DNA of *D. vulgaris* Hildenborough (lane 2), *D. vulgaris* F1 (lane 3), and enrichment cultures (lanes 4 to 12) in medium C with 10 µg Cm ml⁻¹ and 2.5% (lanes 4, 7 and 10), 5% (lanes 5, 8 and 11) or 10% (lanes 6, 9 and 12) sucrose. The DNA samples were restricted with *AccI*, and the blot was hybridized with the 'up' and 'down' probes shown in Fig. 2. Sizes of the relevant *AccI* fragments are indicated on the left. The arrow indicates the 0.95 kb *AccI* fragment which is diagnostic for the gene-replacement product *D. vulgaris* F100.

This integrant, *D. vulgaris* F1, was used for further manipulation.

Effect of sucrose and Cm on the growth of *D. vulgaris* F1

Cm up to 10 µg ml⁻¹ had no severe effects on growth of F1. However, in the presence of both sucrose and chloramphenicol the cell density decreased significantly after one generation (Fig. 4), indicating the occurrence of cell lysis. Addition of sucrose to wild-type *D. vulgaris* samples had no such effect. These results indicated that the *sacB* gene was expressed in *D. vulgaris* F1 and that its

Fig. 4. Sucrose sensitivity of the integrant strain *D. vulgaris* F1. Cells were grown anaerobically at 35 °C in medium C (□), medium C with 10 µg Cm ml⁻¹ (◇), or medium C with Cm and 5% (w/v) sucrose (○). The sucrose was added at the time indicated by the arrow. Cell densities in Klett units are plotted versus time in hours.

product, levansucrase, was toxic to *D. vulgaris* in the presence of sucrose, as has been demonstrated in *E. coli* (Steinmetz *et al.*, 1983), allowing *sacB* to be used as a counter-selectable marker to select for the rare, gene-replacing, second recombination (Fig. 2, ②).

Use of 10 µg Cm ml⁻¹ in both liquid and agar media was found to be the appropriate concentration to select *D. vulgaris* F1 from the wild-type. *D. vulgaris* F1 grew and maintained high plating efficiency, while the wild-type did not grow provided that a cell density of no more than 10^5 cells cm⁻² was used for plating and that no

more than 10^7 cells ml^{-1} (1% inoculum) was used for liquid culture. Hence, Cm at $10 \mu\text{g ml}^{-1}$ and a limited volume of inoculum were used in combination with sucrose in the next step to select for conversion of *D. vulgaris* F1 to the gene replacement mutant F100 and against conversion to wild-type resulting from plasmid excision.

Sucrose and Cm enrichment of the gene-replacing second recombination

D. vulgaris F1 was first grown to saturation in liquid medium C containing Cm to allow more recombination events to happen (Fig. 2, ②) prior to selection of the desired *D. vulgaris* F100. Small inocula (0.1 to 0.2%, v/v) in medium C with Cm and sucrose (Suc) were then grown to saturation in 7 d. Genotypic changes in the population were identified by Southern blot analysis. Deletion of *dcrA* was expected to change the *AccI* fragment size, hybridizing with the 'up' probe, from 0.9 and 4.5 kb in F1 to 0.95 kb in F100 (Fig. 2) and a variable fraction of this F100-specific band was found after the enrichment (Fig. 3, lanes 4 to 12: 30 to 100% of the total population). The rest of the population showed mainly the pattern expected for *D. vulgaris* F1 and occasionally that of the wild-type. Sucrose concentrations of 2.5, 5 and 10% (w/v) were all found to be effective in enrichment of the desired gene-replacing second recombination.

After plating one of the enriched cultures on a medium E plate containing Cm, 117 colonies were obtained. From this plate 30 colonies were randomly selected and grown to saturation in medium C with Cm. Southern blot analysis of their chromosomal DNAs with the *dcrA* flanking region probes indicated 14 of these to have the restriction pattern expected for the *dcrA* gene deletion strain *D. vulgaris* F100 (Fig. 5a, lanes 3, 5, 7, 8 and 10). The remainder had the same restriction pattern as the *D. vulgaris* F1 control (Fig. 5a, lanes 4, 6, 9 and 11). Deletion of the *dcrA* gene was proven unambiguously by stripping the blot shown in Fig. 5(a) of the flanking region probes and re-hybridizing with a *dcrA* probe (Fig. 5b). *D. vulgaris* F100 clearly lacks the *dcrA* gene (Fig. 5b, lanes 3, 5, 7, 8 and 10) which is present in the wild-type (Fig. 5b, lane 1), *D. vulgaris* F1 (lane 2), and the remaining Cm^R Suc^R strains denoted *D. vulgaris* F1SR (Fig. 5b, lanes 4, 6, 9 and 11). The $\Delta dcrA$ strain *D. vulgaris* F100 is *cat*-gene marked (Cm^R) but was stable without Cm selection.

Inactivation of *sacB* by an IS element

The possibility that the *D. vulgaris* F1SR strains harboured a modified *sacB* gene was investigated by stripping and re-probing the blots with the *sacB* gene probe. While, as expected, none of the 14 $\Delta dcrA$ strains (*D. vulgaris* F100) showed hybridization to the *sacB* probe (Fig. 5c, lanes 3, 5, 7, 8 and 10), all of the 16 *D. vulgaris* F1SR strains showed the hybridization, as did their parent *D. vulgaris* F1, indicating that none of these

Fig. 5. Southern blot analysis of DNA from Cm^R Suc^R colonies of *D. vulgaris*. Chromosomal DNAs from *D. vulgaris* wild-type (lane 1), F1 (lane 2), and colony-purified Cm^R Suc^R variants (lanes 3 to 11) were restricted with *AccI*, and the blot was probed with: (a) the up-down probe to identify the desired second recombination products, (b) the *dcrA* probe to confirm deletion of *dcrA*, and (c) the *sacB* probe to identify insertional inactivation of the *sacB* gene. The blot was stripped prior to incubation with each new probe. Sizes of the hybridizing genomic DNA fragments are indicated in kb. The clones showing the predicted pattern of *D. vulgaris* F100 (Δ) and clones showing IS element insertion in *sacB* (*) are indicated. (d) Map indicating a 1.2 kb IS element insertion in the *sacB* gene, explaining the hybridization patterns in (c), lanes 9 and 11.

became sucrose-resistant by plasmid excision. Among these 16 *D. vulgaris* F1SR strains, 10 gave a 3.1 kb *sacB*-hybridizing *AccI* fragment (Fig. 5c, lanes 4 and 6) as did the parent F1 (lane 2), while six of them displayed two *AccI* fragments with a combined size of 4.3 kb (Fig. 5c, lanes 9 and 11), indicating an insertion of a 1.2 kb DNA element with one *AccI* site (Fig. 5d). Cloning and sequencing of the modified *sacB* gene from one of these *D. vulgaris* F1SR strains has indicated that the 1.2 kb fragment is a novel IS element of *D. vulgaris* (unpublished).

Fig. 6. Stability and oxygen sensitivity of the $\Delta dcrA$ strain *D. vulgaris* F100. (a) Southern blot analysis of chromosomal DNAs from *D. vulgaris* wild-type (wt), F100, and a mixed culture of wild-type and F100 grown continuously in medium C without Cm under anaerobic conditions. The number of subcultures of the mixture is indicated above each sample lane. (b) Ratio of F100 to the total population determined by densitometry of the autoradiogram in (a), plotted against subculture number. (c) Southern blot analysis of chromosomal DNAs from a mixed culture of wild-type and F100 in a ratio of 2:1 that was continuously aerated for up to 6 h. The sampling times are indicated above the sample lanes. (d) Ratio of recovered wild-type to F100 cells, determined by densitometry and normalized to 1.0 for time zero, plotted against aeration time. Data are for the autoradiogram in (c) with an initial ratio of wild-type to F100 of 2:1 (□) and for an experiment of which the initial ratio was 1:1 (◆; autoradiogram not shown). The DNAs were restricted with *Eco*RI, and both blots were hybridized with the downstream region probe (Fig. 2).

Changes in oxygen sensitivity of *D. vulgaris* F100 vs wild-type

The growth curve of the F100 strain was identical to that of the wild-type (data not shown), confirming that *dcrA* is not essential for growth under anaerobic conditions. This was also shown in three differential growth experiments in which the wild-type and F100 were co-cultured. The chromosomal DNA ratio of F100 to wild-type remained the same after five sequential subcultures in 7 d, approximately 25 generations (Fig. 6a, b), indicating that F100 was as competitive as the wild-type under anaerobic growth conditions without Cm selection.

To allow the determination of small differences in oxygen sensitivity, a mixture of the two strains was aerated to ensure that both strains received identical aeration treatment. Survival of wild-type vs F100 was determined by restoring anaerobic conditions and by re-growing the diluted samples to saturation before the genomic DNA ratio of the two strains was determined by Southern blot analysis. As shown in Fig. 6(c, d), exposure of a wild-type–F100 mixture to oxygenated medium C resulted in a significant decrease of the ratio of recovered wild-type to F100 cells after 5 h. The same results were obtained with different initial mixing ratios. Even when twice as many wild-type as F100 cells were used, the ratio of recovered wild-type to F100 cells

decreased (Fig. 6d), indicating that the *dcrA* deletion strain F100 is more resistant to inactivation by aeration than the wild-type.

Anaerotaxis behaviour

Anaerotactic movement of *D. vulgaris*, defined as movement away from a liquid–air interface, was first observed by Johnson *et al.* (1995), who suggested that positive aerotaxis (movement towards a liquid–air interface) may also be present, causing the cells to concentrate at an optimal, low oxygen concentration. Motile cells of *D. vulgaris* near the medium–air interface of the microchamber also moved towards the inner area, leaving a clear zone and forming a dense band of cells (not shown). This banding effect was observed for both *D. vulgaris* wild-type and F100. To confirm that this behaviour was due to anaerotaxis, capillary assays were conducted. When a capillary filled anaerobically with a motile cell preparation was opened to the air, the banding appeared within 20 min about 2 mm away from the liquid–air interface. The position shifted further away from the interface with time. No banding was observed in control capillaries which were protected from exposure to air, either by keeping them inside the anaerobic hood or by sealing them anaerobically with petroleum jelly before transfer. These results indicate that band formation was air-dependent. In capillary

Fig. 7. Anaerotaxis assay with change of gas phase. (a) Detail of the capillary tube setup. The capillaries were filled anaerobically and sealed with Plasticine at the medium end. Following exposure to air they were loosely connected with tubing that could be perfused with either O_2 or N_2 . (b) Effects of gas change on anaerobic band shifting of *D. vulgaris*. TY cultures of *D. vulgaris* wild-type (capillaries 1 and 2) and F100 (capillaries 3 and 4) were exposed to air. After the bands were formed (at 20 min) capillaries 2 and 4 were perfused with O_2 (open arrow), while capillaries 1 and 3 were perfused with N_2 (line arrow). Perfusions were halted at 105 min (dashed line), and resumed for capillaries 1, 3 and 4 with a change of gas at 310 min. The distance of the band from the meniscus is plotted against perfusion time.

assays with a gas shift the open ends of capillaries were perfused with pure nitrogen or pure oxygen (Fig. 7a). The changes in banding position from the meniscus were visually recorded with a ruler. Cell bands of both *D. vulgaris* wild-type and F100 strains, formed by exposure to air, shifted further away from the meniscus when oxygen was perfused into the capillaries (Fig. 7b, capillaries 2 and 4) while they remained unchanged in the nitrogen control samples (Fig. 7b, capillaries 1 and 3). When oxygen was later introduced into the nitrogen control samples, the bands moved again further away from the meniscus (Fig. 7b, capillaries 1 and 3).

Northern blot analysis

Recent work by Pianzola *et al.* (1996) has shown that the product of the *rbo* gene, which is present in an operon together with the gene for rubredoxin (*rub*) immediately downstream from *dcrA*, can complement the deleterious effects of *sod* deficiency in *E. coli*. In order to assess whether the increased oxygen resistance of *D. vulgaris* F100 is due to altered *rbo* gene expression, we compared levels of *rbo-rub* mRNA. The major

Fig. 8. Analysis of transcription of the *rbo-rub* operon by Northern blotting. RNA was extracted from *D. vulgaris* wild-type (lanes 1, 3 and 5) and *D. vulgaris* F100 (lanes 2, 4 and 6). Cells were exposed to air for 0 (lanes 1 and 2), 5 (lanes 3 and 4), or 30 (lanes 5 and 6) min prior to RNA extraction. The blot was hybridized with the ^{32}P -labelled insert of plasmid pRbo1 (Brumlik & Voordouw, 1989). The migration positions of 23S, 16S and 5S rRNA are indicated. The numbers below the lanes are the relative levels of transcript obtained by quantification with the phosphor-imaging analyser.

species of *rbo-rub* mRNA detected had a length of 700 nt in both *D. vulgaris* wild-type and F100, in good agreement with the size reported before (Brumlik & Voordouw, 1989). A minor species of larger size (approx. 3000 nt) was also present. The 700 nt transcript was expressed at a fivefold higher level in *D. vulgaris* F100 compared to the wild-type under anaerobic conditions (Fig. 8, lanes 2 and 1; the same result was obtained in two experiments). Exposure to air for 5 or 30 min had no effect on the transcription level of the *rbo-rub* operon in *D. vulgaris* F100, while a 50% increase was recorded for the wild-type (Fig. 8, lanes 3 to 6).

DISCUSSION

Prior to this work, only Rousset *et al.* (1991) had succeeded in using marker-exchange mutagenesis in *Desulfovibrio*, for construction of a [NiFe] hydrogenase gene deletion mutant of *D. fructosovorans*. These authors used electroporation to deliver the non-replicating plasmid and penicillin enrichment for selection of the gene-replacing double recombination. The probable presence of a restriction/modification system, as documented here, prevented use of this approach for *D. vulgaris* Hildenborough. Attempts to electroporate plasmids into *D. desulfuricans* were also unsuccessful (Wall *et al.*, 1993). This may also be due to a restriction problem; for example *D. desulfuricans* strain Norway harbours the restriction endonuclease *DdeI* (Szynter

et al., 1987). The conjugal transfer method with *sacB* counter-selection used here is less affected by host restriction (Schweizer, 1992) and does not require a specific phenotype of the target gene for selection. The physiological function of other genes from *D. vulgaris* Hildenborough which have been cloned and sequenced (Voordouw, 1995) can now, in principle, be tested with this approach.

Use of the *Bacillus subtilis sacB* gene as a counter-selectable marker for gene-replacement mutagenesis has been successful in many other Gram-negative bacteria (Cai & Wolk, 1990; Kamoun *et al.*, 1992; Quandt & Hynes, 1993; Ried & Collmer, 1987; Schweizer, 1992; Schweizer & Hoang, 1995). The *sacB* gene encodes levansucrase, which is toxic to the host in the presence of sucrose (Steinmetz *et al.*, 1983; Gay *et al.*, 1985). The occurrence of spontaneous *sacB* mutations has also been demonstrated in other systems (Cai & Wolk, 1990; Kaniga *et al.*, 1991) and necessitates additional screening either by monitoring the loss of an antibiotic resistance marker or, as we have done, by Southern blotting. We found a high frequency of *sacB* mutations (50% of all $\text{Cm}^R \text{Suc}^R$ colonies), of which 40% were due to insertional inactivation by an endogenous IS element.

The development of an efficient selection system was essential for the success of our method, because conjugal-transfer-based gene replacement requires two homologous recombination events that take place at low frequency, usually between 10^{-9} and 10^{-4} (Blomfield *et al.*, 1991; Kaniga *et al.*, 1991). Unfortunately, *Desulfovibrio* spp. have a high level of endogenous resistance to many antibiotics and many of the commonly used antibiotic resistance genes do not perform well in these bacteria (van Dongen *et al.*, 1994). The Cm^R marker is the only reliable marker found so far for selection in *D. vulgaris* Hildenborough provided that selection of plasmid integrants is done at a Cm concentration of $10 \mu\text{g ml}^{-1}$. At lower concentrations of Cm , for example $5 \mu\text{g ml}^{-1}$ (van den Berg *et al.*, 1989), colonies develop that lack the *cat* gene. Use of a recipient cell density in excess of 10^5 cells cm^{-2} also caused wild-type cells to develop into colonies. These observations are consistent with the fact that Cm is only bacteriostatic and can be detoxified by *cat*-minus anaerobes through a ferredoxin- or NADH-dependent reduction of its aryl nitro group (Argyle *et al.*, 1992; Staudenbauer & Dubbert, 1993). This problem may be aggravated when Km selection against the *E. coli* donor cells containing the *cat* gene on a multicopy plasmid is inefficient.

The deletion of the *dcrA* gene does not affect growth of *D. vulgaris* under anaerobic conditions (Fig. 6a, b). The earlier finding, that the degree of methyl labelling of DcrA's signal transduction domain decreased in response to oxygen (Fu *et al.*, 1994), led to the hypothesis that DcrA allows *D. vulgaris* to recognize oxygen as a chemotactic repellent, i.e. repellents also decrease the degree of methyl labelling of MCPs in the *Enterobacteriaceae* (MacNab, 1987). This hypothesis would be confirmed if deletion of DcrA were to abolish the anaerotactic response (the fleeing from oxygen).

Measurement of the effect of oxygen on *D. vulgaris* chemotaxis is complicated by the fact that oxygen may have pleiotropic effects. Oxygen can react directly with the sulfide formed by sulfate respiration, creating novel gradients, and some sulfate-reducing bacteria, including *D. vulgaris*, have been reported to use oxygen as the terminal electron acceptor (Dilling & Cypionka, 1990). With these reservations in mind, the data indicate that the *dcrA* mutant retains the anaerotactic phenotype (Fig. 7b). This observation rules out DcrA as the sole determining factor in *D. vulgaris* anaerotaxis. Recent work on a novel aerotaxis receptor in *E. coli* has shown that its deletion abolished aerotaxis only after a second gene was also deleted (B. L. Taylor, personal communication). Anaerotaxis of *D. vulgaris* may similarly be determined by multiple genes from the large *dcr* gene family (Deckers & Voordouw, 1994).

The possibility that DcrA serves as a sensor in other cellular processes should also be considered. It is of great interest in this respect that the gene for the oxygen or redox potential sensor DcrA is followed immediately by the gene for Rbo. The transcriptional start of the *rbo-rub* operon is located only 300 bp downstream from the 3' end of the *dcrA* gene and the operon is transcribed in the same direction (Brumlik & Voordouw, 1989; Dolla *et al.*, 1992). A role for Rbo in repair and/or prevention of oxidative damage can be inferred from the work of Pianzola *et al.* (1996), who cloned a DNA fragment from the sulfate-reducing bacterium *Desulfoarculus baarsii* by functional complementation of an *E. coli* strain can only grow anaerobically due to mutations in the *sodA*, *sodB* and *recA* genes. The cloned fragment was found to contain the *rbo* gene, which restored the capacity of aerobic growth. The *rbo* gene from *D. vulgaris*, cloned by Brumlik & Voordouw (1989), also complemented *E. coli* *sod* deficiency (Pianzola *et al.*, 1996). The *rub* gene with which *rbo* forms an operon in both *Desulfoarculus baarsii* and *D. vulgaris* is not required for the complementation in *E. coli*. The Northern blot data indicated that under anaerobic conditions *D. vulgaris* F100 overexpresses *rbo-rub* mRNA about fivefold relative to the wild-type. This could explain the increased oxygen survival of *D. vulgaris* F100. An intriguing possibility is that DcrA is involved in regulation of the expression of the *rbo-rub* operon through a mechanism that represses transcription under anaerobic conditions and allows increased transcription under conditions of oxidative stress. Transcription levels rise somewhat in wild-type cells upon oxygenation (Fig. 8) but do not reach the level found in *D. vulgaris* F100, irrespective of aeration. This may mean that the oxidative stress signal that leads to full induction of *rbo* transcription is not mere aeration and is currently not known. Elucidation of the role and regulatory interactions of the *dcrA-rbo-rub* region in *Desulfovibrio* spp. thus requires more experimentation. The mutagenesis method described here will be a help in future work that focuses on determining the physiological role of Rbo by deletion of its gene from *D. vulgaris*.

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