Recombinant *Klebsiella oxytoca* Strains with Improved Efficiency in Removal of High Nitrate Loads

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Klebsiella oxytoca CECT 4460 removes high nitrate loads from industrial wastewaters without accumulation of nitrite under optimal culture conditions; however, under nonoptimal conditions nitrite accumulates. This situation reflects an in vivo-limited functioning of nitrite reductase in this strain. As a way to overcome this limitation, an increase in the nitrite reductase gene dose in *K. oxytoca* CECT 4460 was considered. To achieve this, we cloned and transferred into this strain the *Klebsiella pneumoniae nasB* gene, which encodes assimilatory nitrite reductase (Lin et al., J. Bacteriol. 176:2551–2559, 1994). The delivery vector was either the wide-host-range plasmid pUPE2, in which the *nasB* gene is expressed from the *Escherichia coli* P_{lac} promoter, or a mini-Tn5-Km vector, which upon random insertion in the host chromosome allowed expression of the *nasB* gene from an unidentified chromosomal host promoter. The effect of the increase in the dose of the *nasB* gene in *K. oxytoca* CECT 4460 on the accumulation of nitrite in the culture medium was tested in two recombinant strains. The results obtained showed that *K. oxytoca* CECT 4460 bearing pUPE2 accumulated 88% less nitrite than the wild-type strain, while the recombinant strain bearing the *K. pneumoniae nasB* gene in the host chromosome showed a 25% lower level of nitrite accumulation in the culture medium than that of the wild type.

Biological removal of nitrogenous compounds from industrial wastewaters with high nitrogen contents has been the focus of many studies, mainly because of its potential advantage and improved removal of these compounds over physical and/or chemical processes (4, 9, 18). Effluents from industrial facilities producing fertilizers, semiconductors, and munitions contain high nitrate loads and are difficult to treat (3, 14, 15, 18, 20, 23). In fact, when these wastewaters reach conventional treatment plants a futile reduction of nitrate to nitrite takes place, which eventually leads to the full collapse of the treatment plants (10). In our efforts to provide microorganisms useful for the biotreatment of wastewaters from the production of dinitroethylene glycol, we isolated a strain of *Klebsiella oxytoca* capable of tolerating high nitrate loads (up to at least 1 M NO₃[−]) and capable of removing from a batch nitrate at concentrations up to 150 mM if sufficient glycerol as a C source was supplied in the culture medium (14). We found that under continuous culture operation the strain could perform in a zone of double nutrient limitation, so that at C/N ratios from 8 to 11 full removal of both C and N sources took place (16). However, when conditions in batch or continuous cultivation were suboptimal, e.g., nitrate load shock, when the C/N ratio was inappropriate, and/or when sucrose was used in the batch instead of glycerol, we observed that part of the nitrate (between 20 and 50%) was reduced to nitrite, which accumulated in the culture medium and eventually led to the arrest of cell growth. Since, during the long-term operation of industrial wastewater treatment plants, it is expected that sudden physicochemical alterations can lead to suboptimal operational conditions, we have explored ways to avoid the accumulation of nitrite. We came to the conclusion that for *K. oxytoca* CECT 4460 the limiting step in the removal of nitrate under nonop-

timal conditions might be the level of in vivo nitrite reductase activity. We then reasoned that a way to bypass this limitation might be to increase the dose of the gene for and expression of the nitrite reductase in this strain. This hypothesis implies that no limitation in the uptake and in the reduction of nitrate to nitrite occurs under nonoptimal conditions.

Subcloning of the *Klebsiella pneumoniae nasB* gene and its transfer to *K. oxytoca* CECT 4460. The assimilatory nitrate reductase (*nasCA*) and nitrite reductase (*nasB*) genes of *K. pneumoniae* are part of a cluster of genes involved in nitrate assimilation and have previously been cloned (11, 12). Hybridization studies with these genes revealed that in *K. oxytoca* the *nasCBA* genes were present as a single copy on the host chromosome and that these genes were clustered in a 17-kb *Hind*III operon as in *K. pneumoniae* (Fig. 1). Given the strong homology of the *nasB* genes of these two strains, we decided to increase the *nasB* gene dose in *K. oxytoca* CECT 4460 via the cloning of the *K. pneumoniae nasB* gene in the former host strain.

To this end two strategies were considered. In the first one, the promoterless *K. pneumoniae nasB* gene was excised from plasmid pVJS615 (11) with *Bam*HI and *Kpn*I and ligated between the same sites in plasmid pJB3Km (1). The ligation mixture was transformed in *Escherichia coli* JM109 (23), and a clone bearing the correct plasmid was selected and called pUPE2 (Fig. 2). In this plasmid, which encodes kanamycin resistance, the *nasB* gene is read from the P_{lac} promoter. The plasmid carrying *mob*⁺ and *tra*, pUPE2, was mobilized from *E. coli* JM109 to *K. oxytoca* CECT 4460 by triparental mating in which *E. coli* HB101 (pRK600) was a helper strain (2, 6–8). Kanamycin-resistant (Km^r) transconjugants of *K. oxytoca* were selected on M8 minimal medium with glycerol as the sole C source, 50 µg of kanamycin per ml, and 20 mM NO₃[−]. The frequency of transconjugants was on the order of 10^{−3} per recipient cell. Because no *lacI* gene encoding the LacI repressor was present in *K. oxytoca* CECT 4460, we concluded that the expression of the cloned *nasB* gene in pUPE2 was constitutive (17). In the second strategy, the *nasB* gene was cloned into a mini-Tn5Km in plasmid pUTKm (6), whose replication de-

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FIG. 1. Hybridization of total DNA of *K. oxytoca* CECT 4460 and its recombinant derivative GP1 with the *nasB* probe. Chromosomal DNA from *K. oxytoca* CECT 4460 (lane 2) and GP1 (lane 1) cells grown on minimal medium was prepared (21), digested with *Hind*III, and hybridized with the *nasB* gene of *K. pneumoniae* labelled with digoxigenin as described before (19). Immunodevelopment of the Southern blot was performed as recommended by Boehringer Mannheim. The size marker was lambda DNA digested with *Hind*III and labelled with digoxigenin (lane M); from top to bottom, the sizes of the bands were 23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kb.

depends on the presence of the PIR protein (8). The cloning was designed so that reading of the *nasB* gene would require the expression of the gene from a host promoter upon the insertion of the mini-Tn5 in the host chromosome. Plasmid pUPE5 (Fig. 2) bears the gene within the borders of the mini-Tn5-Km transposon. This plasmid was propagated in *E. coli* CC118λPIR, and it was used as a delivery system of the *nasB* gene into the *K. oxytoca* chromosome. To this end a triparental mating among *K. oxytoca* CECT 4460 as a recipient, *E. coli* CC118λPIR (pUPE5), and *E. coli* HB101 (pRK600) was performed (7). Since different insertions could give rise to different levels of *nasB* gene expression, 50 random Km^r *K. oxytoca* transconjugants were chosen for further studies and grown in liquid

medium with 40 mM nitrate and 2% (wt/vol) sucrose. Under these conditions the wild-type strain accumulated a high level of nitrite in the culture medium (up to 30 mM). The transconjugants were grown on the same medium, and the transconjugant derivative that accumulated the lowest level of nitrite (about 10 mM in two assays) was selected for further studies (see also below). This clone was called *K. oxytoca* GP1. Southern blot analysis confirmed that *K. oxytoca* GP1 contained two copies of the *nasB* gene, the host *nasCBA* operon, which appeared in a 17-kb *Hind*III fragment, and another gene acquired after the random insertion of the mini-Tn5/*nasB* gene in the host chromosome that was located in a *Hind*III fragment of about 14 kb (Fig. 1).

To determine if the two different recombinant derivatives of *K. oxytoca*, *K. oxytoca* bearing pUPE2 and *K. oxytoca* GP1, exhibited higher nitrite reductase activities than the parental strain, the three strains were grown on M8 minimal medium with 40 mM nitrate and 2% (wt/vol) sucrose or 2% (wt/vol) glycerol as the sole C source (15). Cells in the mid-exponential growth phase were harvested by centrifugation, and cell extracts were prepared by sonication. Cells resuspended in potassium phosphate buffer (100 mM, pH 7.2) were disrupted by sonication (Braun Biotech, Labsonic) with 75-W 20-s pulses delivered for 10 min. The unbroken cells were removed by centrifugation (10,000 × *g* for 10 min at 4°C), and the supernatant was used as a source of protein for enzymatic assays. The levels of nitrate reductase and nitrite reductase activities in these extracts were determined as described elsewhere (5). The results obtained are shown in Table 1. As expected, the levels of nitrate reductase were equally high in the three strains, although in the cells grown on glycerol the levels (110 ± 15 mU/mg of protein) (mean ± standard error of the mean) were nearly twice as high as those in the cells grown on sucrose (40 to 60 mU/mg of protein). The results presented are averages of four independent determinations. The levels of nitrite reductase for the two recombinant strains were significantly higher than those determined for the wild-type strain. For *K. oxytoca* bearing pUPE2 these levels were three- to fourfold higher than for the wild-type strain, while for *K. oxytoca* GP1 the level of activity of this enzyme was about threefold higher than for the wild-type strain. These results show that the recombinant strains exhibited a higher dose of nitrite reductase than the wild-type strain, even though they had similar nitrate reductase levels, as expected from the lack of alteration of the dose of the *nasCA* genes.

Effect of extra copies of the *nasB* gene in *K. oxytoca* CECT 4460 on the accumulation of nitrite and ammonium in the culture medium. To determine the effect of the extra *nasB* gene dose in *K. oxytoca* on nitrate removal and nitrite accumulation, cells of *K. oxytoca* CECT 4460, *K. oxytoca* CECT 4460 (pUPE2), and *K. oxytoca* GP1 were grown in batch cultures on

FIG. 2. Maps of plasmids pUPE2 and pUPE5. Relevant restriction sites, antibiotic markers, origin of transfer (*oriT*), and origin of replication (*oriV*) are indicated.

TABLE 1. Nitrate reductase and nitrite reductase activities in cell extracts of wild-type *K. oxytoca* CECT 4460 cells and recombinant derivatives bearing the *K. pneumoniae nasB* gene

Strain	Activity (mU/mg of protein) for cells grown with:			
	Glycerol as sole C source		Sucrose as sole C source	
	Nitrate reductase ^a	Nitrite reductase ^b	Nitrate reductase ^a	Nitrite reductase ^b
<i>K. oxytoca</i>	95 ± 5	11 ± 1	40 ± 4	10 ± 3
<i>K. oxytoca</i> bearing pUPE2	100 ± 10	40 ± 3	60 ± 6	34 ± 2
<i>K. oxytoca</i> GP1	115 ± 15	30 ± 2	50 ± 5	32 ± 6

^a Nitrate reductase activity was determined in 1 ml of assay mixture containing 100 mM potassium phosphate (pH 7.0), 0.9 mM methyl viologen, 1 mM NAD⁺, and 0.25 mM NADH. After the addition of a cell extract, the reaction was started by the addition of 0.1 ml of the reducing agent sodium dithionite (160 mg/ml). Samples were taken at various intervals, and the reaction was stopped by vigorous swirling. Then, the amount of nitrite produced was determined (22). One unit was defined as 1 μ mol of nitrite produced per min.

^b Nitrite reductase activity in cell extracts was determined essentially by the method of Coleman et al. (5). One unit was defined as 1 μ mol of nitrite consumed per min.

minimal medium with 40 mM KNO₃ and sucrose (3% wt/vol). In the assays, growth measured as turbidity of the culture (Fig. 3A) and the concentrations of nitrate (not shown), nitrite, and ammonium in the culture medium were determined (Fig. 3C and D). We also determined nitrite reductase and nitrate reductase activities during growth (13) (Fig. 3B). We observed a sequential pathway for the reduction of nitrate to ammonium in the three strains in four independent assays. The results of one assay for *K. oxytoca* CECT 4460 and *K. oxytoca* bearing

FIG. 3. Effect of *nasB* gene copy number increase on the accumulation of nitrite and ammonium in the culture medium. *K. oxytoca* CECT 4460 (○) and *K. oxytoca* CECT 4460 bearing pUPE2 (●) were grown on minimal medium, with 40 mM KNO₃ and 3% (wt/vol) sucrose at 30°C for 24 h. Growth (A), nitrite reductase activity (B), nitrite concentration (C), and ammonium concentration (D) were determined during the growth of both strains. Nitrite concentration was determined by the method of Snell and Snell (22). Ammonium concentration was determined enzymatically by using a commercial kit from Boehringer Mannheim (Ref. 1112732). Nitrite reductase activity was determined in permeabilized cells as described by Neubauer and Götz (13).

pUPE2 are shown in Fig. 3. The results obtained with *K. oxytoca* GP1 were similar to those obtained with *K. oxytoca* bearing pUPE2 but with some differences which are noted in the text. The wild-type *K. oxytoca* and *K. oxytoca* bearing pUPE2 showed similar growth curves (Fig. 3A). The three strains consumed nitrate at similar rates (0.7 ± 0.1 g of NO₃⁻/g of protein per h), and the nitrate was fully consumed after 18 h (not shown). Nitrite accumulated after the reduction of nitrate (Fig. 3C) and was eventually reduced to ammonium, which again accumulated transiently in the culture medium (Fig. 3D). The highest levels of nitrite in the culture media of the wild-type and the recombinant strains were detected 18 h after the cultures were set up. In the supernatants of the culture of the wild-type strain up to about 18 mM nitrite was found. In contrast, when *K. oxytoca* CECT 4460 bore plasmid pUPE2 the level of nitrite in the medium was lower (2 mM) (Fig. 3B). For strain GP1 the highest level of nitrite was 10 mM (not shown). The highest levels of ammonium in the culture medium were detected 22 h after the cultures were set up. In the supernatants of the culture of the wild-type strain up to about 5 mM ammonium was found. In contrast, when *K. oxytoca* CECT 4460 bore plasmid pUPE2 the level of ammonium in the medium was higher, 13 mM (Fig. 3D), and the level was 12 mM for *K. oxytoca* GP1 (not shown). However, after some time the ammonium was fully consumed (Fig. 3D).

The level of nitrite reductase in the three strains was determined at various times. At the beginning of culture the activity was highest, and the activity had a tendency to decrease with time, reaching about 30 to 10% of the maximum at the end of the assay (Fig. 3B). For *K. oxytoca* CECT 4460 bearing pUPE2 the level of nitrite reductase was always higher than that determined in the wild-type *K. oxytoca* CECT 4460 strain (Fig. 3B). Nitrite reductase activity showed a maximum level of 11 ± 0.1 mU/mg of protein, and the level was about threefold higher in the clone bearing the pUPE2 plasmid than in the wild type. The nitrate reductase activities of the three strains were also determined in permeabilized cells and showed a maximum activity of 50 ± 7 mU/mg of protein (not shown).

In summary, our results show that an increase in the dose of the *nasB* gene in *K. oxytoca* suffices to overcome nitrite accumulation under suboptimal growth conditions. The improved strain represents a more reliable one for the biotreatment of wastewaters with high nitrate loads.

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