Identification and characterization of the *Staphylococcus carnosus* nitrate reductase operon

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**Abstract** Physiological and genetic characterization of *Staphylococcus carnosus* nitrate reductase-negative mutants led to the identification of the nitrate reductase operon, *narGHJI*. Transcription from the *nar* promoter was stimulated by anaerobiosis, nitrate, and nitrite. This is in accordance with the nitrate reductase activities determined with benzyl viologen as electron donor. However, in the presence of oxygen and nitrate, high transcriptional initiation but low nitrate reductase activity was observed. Since the *ab* complex of the nitrate reductase formed during anaerobic growth was insensitive to oxygen, other oxygen-sensitive steps (e.g., post-transcriptional mechanisms, molybdenum cofactor biosynthesis) must be involved. The nitrate-reducing system in *S. carnosus* displays similarities to the dissimilatory nitrate reductases of *Escherichia coli*. However, in the *S. carnosus nar* promoter, no obvious Fnr and integration host factor recognition sites are present; only one site that is related to the *E. coli* NarL consensus sequence was found. Studies to determine whether the *E. coli* proteins NarL and Fnr are functional at the *S. carnosus narGHJI* promoter indicated that the promoter is not functional in *E. coli*.

**Key words** *Staphylococcus carnosus* · *narGHJI* operon · Nitrate reductase · NarL · Fnr

**Introduction**

After oxygen, the most widely used alternative electron acceptor is nitrate. The enzyme that catalyzes the energy-gaining reduction of nitrate to nitrite is well characterized in *Escherichia coli*. The membrane-bound dissimilatory nitrate reductase (NRA) is an enzyme complex composed of three subunits, α, β, and γ. The corresponding genes are organized in an operon, *narGHJI*, together with a fourth gene, *narJ*, which encodes the δ-subunit. This protein is not part of the NRA nitrate reductase, but is most likely required for assembly of the native enzyme complex (Dubourdieu and DeMoss 1992). The γ-subunit, a *b*-type cytochrome, receives electrons from the quinone pool. The electrons are then transferred via the iron-sulfur clusters of the β-subunit to the molybdenum cofactor bound to the α-subunit (Blasco et al. 1989).

Expression of the *narGHJI* operon in *E. coli* is positively regulated by two *trans*-acting factors, Fnr and NarL-phosphate (NarL-P). The Fnr protein is a one-component sensor/regulator that controls gene expression in response to oxygen deprivation. The *narGHJI* transcriptional initiation site is preceded by an Fnr binding site centered at −41.5 (Walker and DeMoss 1991). The NarL protein is the response regulator of a two-component system that signals the presence of nitrate. NarL-P binds to two regions of the *narGHJI* promoter (−81 to −120 and −180 to −215) that contain heptameric sequences defined as specific NarL-binding sequences (Dong et al. 1992). The integration host factor (IHF) is also required for induction of this operon (Rabin et al. 1992; Schröder et al. 1993).

In contrast, the second dissimilatory nitrate reductase of *E. coli*, NRZ, encoded by the *narZYWV* operon, is produced constitutively in small amounts. NRZ is biochemically, immunologically, and genetically very similar to NRA (Blasco et al. 1990).

*Staphylococcus carnosus* is traditionally used as a starter culture in the production of raw fermented sausages and dry-cured ham. The reduction of nitrate to
nitrite is the main function of this organism in the curing process. We have shown that S. carnosus reduces nitrate to ammonia in a two-step mechanism (Neubauer and Götz 1996). First, nitrate is reduced to nitrite, evidently by a dissimilatory enzyme. Nitrite accumulates in the growth medium and is further reduced by a dissimilatory NADH-dependent nitrite reductase. Nitrite reduction only occurs in the absence of nitrate (Neubauer and Götz 1996).

In the present work, we characterized transposon-insertion mutants defective in nitrate reduction and cloned and sequenced the genes encoding the dissimilatory nitrate reductase of S. carnosus. The influence of oxygen, nitrate and nitrite on narG transcription and nitrate reductase activity was investigated.

Materials and methods

Bacterial strains and plasmids

Staphylococcus carnosus TM300 (Schleifer and Fischer 1982; Götz et al. 1983) was used for isolation of chromosomal DNA, as cloning host for the staphyloccocal promoter probe vector pPS44 (Peschel et al. 1993), and as host for pCX15 (Wieland et al. 1995), a plasmid in which the Staphylococcus hyicus lipase gene is under transcriptional control of the xyIA promoter and XylR. pCX15 derivates were constructed as follows. The major part of the S. hyicus lipase gene was cut out from pCX15 with BamHI and NarI. The NarI site was blunt-ended to obtain an HpaI-compatible end for ligation with the polymerase chain reaction (PCR)-amplified narHI DNA region (containing the original ribosome binding site sequences) digested with BamHI and HpaI, sites for which were introduced via the PCR primers. The constructed plasmid was named pCXΔy. Plasmid pCXΔy, which contains the PCR-amplified narI gene, was constructed similarly. To destroy the intact narI gene, pCXΔy was cleaved with BglII and religated. This resulted in a deletion of 522 nucleotides such that the first 20 codons of narI were removed. 13 additional codons and then the stop codon. This construct was designated pCXΔy. Vector pCX15 was cleaved with BamHI and NarI, treated with the Klenow fragment, and religated. This resulted in a major part of the lipase gene was deleted, named pCX15Δlp. Plasmid pTV1Ts (Youngman et al. 1989) was used for introducing Tn917 into S. carnosus. In plasmid pRB473, a derivative of pRB373 (Brückner 1992), a partial gene library of S. carnosus TM300 narI (this study) was constructed using E. coli DH5 (Hanahan 1983) as host. Plasmids pACYC184 (Chang and Cohen 1978) and pUC18 (Veira and Messing 1982) were used for the construction of partial gene libraries of S. carnosus wild-type in E. coli DH5 and E. coli SURE (Stratagene), respectively. DNA was subcloned in E. coli SURE using pUC18 and pBluescriptII KS+ (Stratagene) for sequencing. To obtain modified DNA, E. coli DH5s (Hanahan 1983) was transformed with the promoter probe shuttle vector pRB384 (Brückner 1992) and derivatives. The plasmids were subsequently transferred into the isogenic E. coli strain M182 and its fnr derivative (Darwin et al. 1993), and into the isogenic NarL− strain, JCB3879, and the NarL+ strain, JCB38790. E. coli JCB38790 was prepared by transducing strain JCB38788 (Grove et al. 1996) to kanamycin (kan) resistance with phage P1 propagated on the pcrB:kun strain RP7947 (Liu and Park 1989). Strain JCB38790 was then prepared by transducing JCB38789 to tetracycline resistance with phage P1 propagated on strain RK5278, as described by Page et al. (1990).

Growth conditions

For growth studies, strains were cultivated at 37°C in modified basic medium (B-medium; Götz and Schumacher 1987; Neubauer and Götz 1996). Cells were grown aerobically with vigorous shaking in flasks filled only to 8–16% of their maximal volume. Cells were grown anaerobically by adding Oxyrase (20 ml/l medium; Oxyrase, Mansfield, Ohio) to the cultures and incubating in screw-capped bottles with stirring (100 rpm). Since pCX15 is a xylose-inducible and glucose-repressible expression vector, the complementation studies were performed in modified B-medium without glucose but with 0.5% xylose. For induction studies, S. carnosus wild type was cultivated aerobically or anaerobically with or without NaNO3 (20 mM) or NaNO2 (2 mM), harvested in exponential growth phase, and used for determination of benzyl viologen-nitrate reductase activity.

Analytical determinations

The nitrite concentration was measured by a colorimetric assay as described by Richardson and Nason (1957) and modified by Showe and Demoss (1968). The protein concentration was measured by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Nitrate reductase activity

Nitrate reductase activity was assayed as benzyl viologen-nitrate reductase activity as described by Neubauer and Götz (1996).

Transposon mutagenesis

S. carnosus was mutagenized with Tn917 as described by Fast et al. (1997). The Tn917 insertion mutants obtained were screened in microtiter plates for nitrate reduction different than that of the wild-type. In this assay, nitrite accumulation in B-medium with 10 mM NaNO3 was determined after overnight incubation.

DNA preparation and purification of DNA fragments

Chromosomal DNA from S. carnosus TM300 (wild-type) and S. carnosus Tn917 insertion mutants narI and nar9 (this study) was isolated by the method of Marmur (1961). Staphylococcal plasmid DNA was prepared by the cleared-lysate method (Novick and Bouachaud 1971). Cells were lysed with lysostaphin (10 μg/ml; Sigma). Plasmid DNA was separated from chromosomal DNA by centrifugation in a CsCl density gradient and subsequently dialyzed. Plasmid DNA from E. coli strains was isolated using the NUCLEOBOND AX PC-Kit 100 for plasmid and cosmid purification (Machery-Nagel, Düren, Germany). Chromosomal DNA fragments >5 kb used to construct partial gene libraries were extracted from agarose gels using the Biotrap BT1000 system (Schleicher and Schuell, Dassel, Germany). Other DNA fragments were extracted using Qiagen (Qiagen, Hilden, Germany).

Transformation

Plasmids were introduced into S. carnosus by protoplast transformation (Götz and Schumacher 1987) or by electroporation (Augustin and Götz 1990). Host strains of E. coli were transformed with recombinant plasmids by electroporation (Dower et al. 1988).

Nucleotide sequencing and analysis

Double-stranded DNA was sequenced using the dye dideoxy chain-termination procedure, the Pharmacia AutoRead Sequencing kit, and the A.L.F. DNA Sequencer (Pharmacia). Oligonucleotides labeled with fluorescein were obtained from Pharmacia. Chromosomal DNA of mutants narI and nar9 was sequenced directly using the LI-COR DNA Sequencer (Lincoln Co-operation), the thermo sequenase fluorescent labeled primer cycle sequencing kit (Amer-