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Contribution of nitric oxide synthase from coagulase-negative staphylococci to the development of red myoglobin derivatives

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ABSTRACT

As part of the microbial community of meat or as starter cultures, coagulase-negative staphylococci (CNS) serve several essential technological purposes in meat products, such as color development through the reduction of nitrate to nitrite. As the safety of nitrite as an additive has been questioned, we explored the potential of CNS to develop red myoglobin derivatives such as oxymyoglobin and nitrosomyoglobin. Nitrosoheme was extracted to evaluate NO production. This production could be due to a nitric oxide synthase (NOS) activity. In all CNS strains, a *nos* gene was identified. The NOS sequences deduced were highly conserved within CNS. A phylogenetic tree based on the NOS sequences revealed that the strains within species were clustered. Ninety-one percent of the strains, whatever the species, were able to form red myoglobin derivatives in aerobic conditions, but a high variability was observed between strains within species. However, NO production was low as nitrosomyoglobin represented 8% to 16% of the red pigments according to the species. Formation of oxymyoglobin, especially under aerobic conditions, was substantial, but varied greatly within species. The mechanism involved in the formation of oxymyoglobin could rely on staphylococcal reductases and remains to be explored.

1. Introduction

Coagulase-negative staphylococci (CNS) are particularly common in foods of animal origin, such as dairy and meat products (Coton et al., 2010; Irlinger, 2008). CNS, as well as lactic acid bacteria (LAB), are predominant microorganisms in meat products and are of technological interest (Lücke, 2000). The diversity of *Staphylococcus* species in meat products, especially in traditional raw-fermented sausages or dried meats, is wide and related to the variety of products and manufacturing processes. Overall, however, the species *Staphylococcus xylosus*, *Staphylococcus equorum* and *Staphylococcus saprophyticus* are frequently dominant, and *Staphylococcus carnosus*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus lentus*, *Staphylococcus pasteuri*, *Staphylococcus sciuri*, *Staphylococcus simulans*, *Staphylococcus succinus*, *Staphylococcus vitulinus*, and *Staphylococcus warneri* (non-exhaustive list) are commonly present (Blaiotta et al., 2004; Corbière Morot-Bizot et al., 2006; Coton et al., 2010; Iacumin et al., 2012; Leroy et al., 2010; Marty et al., 2012; Ratsimba et al., 2017). Among all these species, only two, *S. carnosus* and *S. xylosus*, are commonly used as meat starter cultures in order to improve the safety and stability of the products, and to enhance their sensory characteristics (Leroy et al., 2006; Leroy et al., 2016; Talon and Leroy, 2014). In particular, *S. carnosus* and *S. xylosus* improve the development and stability of the characteristic red color of fermented meat products. In these products, nitrate and/or nitrite is added as curing salt for the development of the color of these products, and *S. carnosus* and *S. xylosus* reduce nitrate to nitrite through their nitrate reductase activity (Talon et al., 1999). Nitrite is then chemically converted to nitric oxide (NO), which binds to the myoglobin ferrous heme-iron to form the stable red nitrosomyoglobin pigment (Gøtterup et al., 2007, 2008).

The use of curing salts is regulated by law, with specific indications in the USA (Code of Federal Regulations, 2016) and Europe (Commission Regulation [EU], 2011). Nevertheless, the safety of nitrite has been questioned. This additive can react with the secondary amine groups of muscle proteins and may give rise to unwanted and toxic N-nitroso compounds such as nitrosamines in the gastrointestinal tract (De Mey et al., 2015; Honikel, 2008). The meat industry is therefore looking for alternatives to decrease nitrate and nitrite in the production

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of cured meat products.

S. xylosus converts metmyoglobin to nitrosomyoglobin in culture medium and in meat products without addition of nitrate or nitrite (Li et al., 2013, 2016; Morita et al., 1998). This NO production has been suggested to be linked to nitric oxide synthase (NOS) activity. Bacterial NOSs catalyze oxidation of arginine, which generates NO and citrulline using cellular reductase partners as electron donors (Crane et al., 2010; Gusarov et al., 2008). *Staphylococcus* NOS has been mainly studied in *S. aureus* (Bird et al., 2002; Chartier et al., 2006; van Sorge et al., 2013). Recently, we demonstrated NOS-dependent NO production in the culture medium of the *S. xylosus* C2a, strain by characterization of the corresponding *nos* deletion mutant (Ras et al., 2017). The *nos*-encoding gene is present among all *Staphylococcus* genomes sequenced (Sapp et al., 2014) and has been identified from 30 *Staphylococcus* species, including 25 coagulase-negative species (UniProtKB database, release 2017_03). NOS activity has been investigated among different species of CNS, but was only revealed for one strain of *S. haemolyticus* (Sánchez Mainar and Leroy, 2015; Sánchez Mainar et al., 2014).

In this study, we explored the diversity of the *nos* gene in five species of CNS isolated from meat. We evaluated the potential of these species to develop a red coloration *via* their ability to convert metmyoglobin to red myoglobin derivatives such as oxymyoglobin and nitrosomyoglobin. Assessment of nitrosomyoglobin formation was investigated by evaluation of the nitrosoheme.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A total of 113 strains of CNS, all isolated from meat and meat products from various origins, were used in this study. The *S. xylosus* C2a strain, for which the NOS-mediated NO production was demonstrated, was used as reference (Ras et al., 2017). The collection was composed of *S. carnosus* (16 strains), *S. xylosus* (30 strains), *S. saprophyticus* (27 strains), *S. equorum* (34 strains), and *S. warneri* (6 strains). Strains were routinely cultured in tryptic soy broth (TSB, Difco) under aerobic conditions (1:10 volume to flask ratio, 150 rotation per minute) at 30 °C.

2.2. DNA extraction, primers, PCR and sequencing

Genomic DNA was prepared from overnight cultures. Briefly, cells were resuspended in Tris-EDTA-sucrose buffer containing 0.1 mg/mL lysostaphin (Sigma-Aldrich) and incubated for 30 min at 37 °C. Cells were lysed with sodium dodecyl sulfate and treated with RNase A. Following extraction with phenol-chloroform-isoamyl alcohol (25/24/1) and chloroform, DNA was precipitated with ethanol and resuspended in Tris-EDTA buffer (pH 8.0). Amplification of *nos* was performed using primers designed for each species (Table 1) and GO Taq DNA polymerase (Promega). PCR was carried out using the following conditions: initial denaturation step at 94 °C for 5 min, primer annealing at 55 °C for *S. xylosus*, 54 °C for *S. equorum* and *S. warneri* and 53 °C for *S. carnosus* and *S. saprophyticus* for 30 s, elongation step at 72 °C for 1 min and denaturation at 94 °C for 30 s. Overall, 25 cycles were performed. A final elongation step at 72 °C for 5 min was conducted. PCR products were visualized by 1% agarose gel electrophoresis with SafeView staining (Applied Biological Materials), and imaged on a Gel Doc 2000 (Bio-Rad). PCR products were purified using QIAquick PCR Purification Kit (Qiagen). The sequencing of purified fragments was performed by GATC Biotech (Mulhouse, France). The generated sequences were edited with Chromas Lite (version 2.6, Technelysium Pty Ltd, South Brisbane, Queensland, Australia). The *nos* forward and reverse sequences of the 113 strains were assembled using online CAP3 (Huang and Madan, 1999).

2.3. Phylogenetic analysis

The nucleotide sequences of *nos* genes were converted into amino acid sequences and aligned with MACSE (Ranwez et al., 2011). A maximum likelihood-based phylogenetic tree was built with the online PHYML server (version 3.0) (Guindon et al., 2010). A model selection procedure based on the Akaike information criterion selected an LG amino acid substitution model and a Γ distribution to take into account variation of mutation rate among positions (Le and Gascuel, 2008). A near-neighbor interchange (NNI) branch swapping process was used during tree optimization. A thousand bootstrap replicates were obtained to assess the robustness of the inferred phylogenetic relationships. The amino acid residues making up the active site were determined on the basis of the analysis of Pant et al. (2002) using the *S. aureus* NOS as template. The data were submitted to WebLogo (weblogo.berkeley.edu/logo.cgi) to generate sequence profiles of the active site.

2.4. Metmyoglobin conversion and nitrosoheme formation

These assays were performed as described by Ras et al. (2017). Briefly, overnight cultures were inoculated at an $OD_{600\ nm}$ of 0.5 in TSB supplemented with freshly prepared metmyoglobin solution (2 mg/mL). Cultures and control samples (TSB-metmyoglobin without bacteria) were incubated either under limited oxygen (covered with mineral oil and without stirring) or under aerobic conditions. After 24 h of incubation at 30 °C, culture supernatants were used to measure the absorbance spectrum between 500 and 600 nm (BioMate 3, Thermo Fisher Scientific) using TSB-metmyoglobin as blank. Then, to determine the production of nitrosomyoglobin in the culture supernatants, the nitrosoheme was extracted with acetone and absorbance was acquired between 450 and 640 nm (V-770 UV/visible, Jasco) as described by Yu et al. (2016). Experiments were carried out in three independent biological replicates.

Table 1
Oligonucleotides designed to amplify the *nos* gene.

Name	Sequence (5′ to 3′)	Fragment size	Targeted species
SX*nos*F4	AAGCGAAATCCTTTATCGACAC	1051 bp	*S. xylosus* based on C2a sequence (LN554884)
SX*nos*R4	TGAAATGGACAACCAGTAGCTT	(1065)[a]	
SS*nos*F1	CCAAAATGGCATGGAGAAAT	910 bp	*S. saprophyticus* based on sequence ATCC15305 (NC_007350.1)
SS*nos*R1	TGGAATGGACAACCTGAGTCT	(1065)	
SQ*nos*F1	TCAAACAAATGGCACGTATGA	962 bp	*S. equorum* based on KS1039 sequence (NZ_CP013114.1)
SQ*nos*R1	AATGGAATGGACAACCCGTA	(1065)	
SW*nos*F1	GCAACAACATTTATCCAAACGA	1048 bp	*S. warneri* based on SG1 sequence (NC_020164.1)
SW*nos*R1	GGAAAGGGCACTTTGTCTCA	(1068)	
SC*nos*F1	AAATAAACGAAAGCGGGACA	968 bp	*S. carnosus* based on TM300 sequence (NC_012121.1)
SC*nos*R1	AAAAGGGCACCCTTGTTTTT	(1074)	

[a] Numbers in brackets indicate the total length of the gene of each species (bp).