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Ferritin, an iron source in meat for *Staphylococcus xylosus*?

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ABSTRACT

Staphylococcus xylosus is frequently isolated from food of animal origin. Moreover, this species is one of the major starter cultures used for meat fermentation. Iron is a key element for growth and survival of bacteria. Meat is particularly rich in haemic (myoglobin and haemoglobin) and non-haemic (ferritin and transferrin) iron sources. Ferritin is a storage protein able to capture large quantities of iron. It is highly resistant to microbial attack and few microorganisms can use it as an iron source. Surprisingly, we found that the *S. xylosus* C2a strain grows in the presence of ferritin as a sole iron source. A three-cistron operon was highly overexpressed under ferritin iron growth conditions. We generated a deletion–insertion in the first gene of the operon and evaluated the phenotype of the mutant. The mutant showed decreased growth because it was less able to acquire iron from ferritin. Transcriptional analysis of the mutant revealed downregulation of several genes involved in the response to oxidative stress. This study characterized for the first time the capacity of a *Staphylococcus* to use iron from ferritin and revealed that a potential reductive pathway was involved in this acquisition. We hypothesize that this ability could give an advantage to *S. xylosus* in meat products.

1. Introduction

Staphylococcus xylosus belongs to the vast group of coagulase-negative staphylococci (CNS) and lives naturally on the skin and mucous membranes of mammals (Dordet-Frisoni et al., 2007; Götz et al., 2006; Nagase et al., 2002). So it occurs in foods of animal origin, such as meat, milk, fermented meat products and cheeses (Coton et al., 2010; Leroy et al., 2010). It is also used widely as a starter culture for meat fermentation (Talon and Leroy, 2011). *S. xylosus* has the ability to grow on meat and to survive during fermentation (Vermassen et al., 2014, 2016) and for long periods of ripening during sausage manufacturing (Blaiotta et al., 2004; Corbière Morot-Bizot et al., 2006). The capacity of *S. xylosus* to use meat substrates has contributed to its adaptation to this environment. Meat is an iron-rich substrate but the bioavailability of iron is poor as it is complexed within heme as a cofactor for myoglobin or haemoglobin, or bound within ferritin or transferrin (Linder et al., 1981). Non-heme iron can range from 15 to 70% of total iron (about 2 to 5 μg/g), depending on the type of meat (Lombardi-Boccia et al., 2002; Pereira and Vicente, 2013). The source of iron in meat used by *S. xylosus* is unknown.

For almost all bacteria, iron is a key element, required for survival and for many cellular processes (Wandersman and Delepelaire, 2004). Staphylococci have evolved a plethora of mechanisms to acquire iron from the host, including the elaboration of siderophores, the utilization of exogenous siderophores, the acquisition of iron from heme and haemoproteins, and the uptake of inorganic free iron (Beasley and Heinrichs, 2010; Haley and Skaar, 2012; Sheldon and Heinrichs, 2012). Among these mechanisms, two seem to be present only in pathogenic staphylococci. The first is the iron-regulated surface determinant (Isd) system, which allows the capture of heme from haemoglobin and its transfer into the cytoplasm, and has only been found in *Staphylococcus aureus* and *Staphylococcus lugdunensis* (Skaar and Schneewind, 2004; Zapotoczna et al., 2012). The second system has only been described for *S. aureus* and ensures the synthesis and transport of the staphyloferrin B (Dale et al., 2004). Most studies on iron acquisition have concerned the pathogenic *S. aureus* species and have focused on the acquisition of this essential element from haemoglobin or transferrin (Hammer and Skaar, 2011; Skaar et al., 2004). To our knowledge, nothing is known about ferritin iron acquisition by staphylococci.

Ferritin is a ubiquitous intracellular storage protein for iron. It is composed of 24 subunits, which self-assemble through non-covalent interactions into a hollow spherical shell, and contains up to 4500 iron atoms, making it a potentially rich iron source for bacteria. Ferritin uses O_2 or H_2O_2 to catalyse oxidation of Fe(II) to Fe(III) and stores Fe(III) as an iron mineral core similar in structure to ferrihydrite (Bou-Abdallah, 2010). Ferritin is a remarkable iron source, but to acquire it bacteria must be able to remove it from this stable supramolecular complex.

There are few published reports describing microorganisms able to acquire iron from ferritin and even fewer characterizing the mechanisms. Ferritin iron acquisition in *Listeria monocytogenes* involves a surface-associated ferric reductase (Deneer et al., 1995) and *Burkholderia cenocepacia* uses a mechanism requiring proteolytic

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degradation of ferritin by a serine protease (Whitby et al., 2006). In *Bacillus cereus*, the surface protein Ilsa is absolutely required for ferritin iron acquisition (Daou et al., 2009). Recently, it was shown that this protein acts as a ferritin receptor, which causes the aggregation of ferritin on the surface of *B. cereus* (Segond et al., 2014). These authors have proposed a model of ferritin iron acquisition in *B. cereus* in which the surface protein enhances release of iron, uptake of which is then assisted by the siderophore bacillibactin (Segond et al., 2014). The opportunistic pathogen yeast *Candida albicans* captures ferritin though the invasion-like adhesin Als3, which is associated with hyphal morphology and uses a reductive pathway for iron uptake (Almeida et al., 2008). Moreover, *C. albicans* mediates acidification of the growth medium leading to dissociation of ferritin (Almeida et al., 2008).

Ferritin sequesters intracellular iron and is found in all tissues (Arosio et al., 2009) and so is one of the major sources of iron in meat as being a part of non-heme iron (Linder et al., 1981). Our preliminary data indicated that *S. xylosus* may acquire iron from this source and in a microarray study of the *S. xylosus* strain C2a, we identified three significantly upregulated genes that could be involved in the acquisition of iron from ferritin (our unpublished data). The homology-based annotation of these genes revealed that they can encode an oxidoreductase, a monooxygenase and a transporter, respectively. These genes have not yet been studied for any bacteria for their implication for iron acquisition. We have characterized the growth of *S. xylosus* in the presence of ferritin as a sole iron source and show that these three genes are transcribed as an operon encoding potential reductive uptake involved in ferritin iron acquisition.

2. Materials and methods

2.1. Bacterial strains and growth conditions

S. xylosus and *Escherichia coli* strains and plasmids used in this study are listed in Table 1. *S. xylosus* strains were serially precultured in polycarbonate Erlenmeyer flasks for 24 h at 30 °C with orbital shaking of 150 rpm in Mx0 medium unless otherwise indicated. Mx0 contained (per litre): 1 g Na_3-citrate $2H_2O$, 7 g $Na_2HPO_4 \cdot 2H_2O$, 3 g KH_2PO_4, 1 g NaCl, 1 g KCl, 4 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.0147 g $CaCl_2 \cdot 2H_2O$, 4.6 mg nicotinic acid, 2 mg thiamine hydrochloride, 0.012 mg biotin, 5 g glucose, and 6 g casamino acids. Iron-starved strains were subcultured at 30 °C in medium supplemented or not with horse spleen ferritin (0.25, 0.5, 1 or 5 μM) or $FeSO_4$ (5, 50, 250 or 500 μM). When needed, agar (Difco™ agar, Difco Laboratories, Detroit, MI, USA), containing or not bromocresol green as pH indicator, was added. A fresh solution of ferritin was passed through Amicon ultrafilter units 10 kDa (Merck Millipore, Molsheim, France) to eliminate traces of released iron. *E. coli* was routinely grown under aerobic conditions in Luria-Bertani broth (Difco Laboratories) at 37 °C. Cell growth was monitored by reading optical density at 600 nm. Antibiotics were added at the following concentrations when necessary: for *E. coli*, 100 μg/mL ampicillin; for *S. xylosus*, 20 μg/mL chloramphenicol, and 2.5–10 μg/mL erythromycin. All chemicals were from Sigma-Aldrich (Saint-Quentin Fallavier, France).

2.2. Bioscreen assay

Precultures of *S. xylosus* strains in Mx0 were diluted in fresh Mx0 media supplemented or not with ferritin or $FeSO_4$ to an $OD_{600\ nm}$ of 0.04. Cells were grown at 30 °C in 100-well microtitre plates with continuous shaking in a Bioscreen C plate reader (Labsystems France, Les Ulis, France). The $OD_{600\ nm}$ was measured every 30 min. Three independent experiments were done for each condition.

When needed, a protease inhibitor cocktail (P8465) was prepared according to the manufacturer's instructions (Sigma-Aldrich) and added. This cocktail contains AEBSF, sodium EDTA, bestatin, pepstatin A and E64 and has a broad specificity against serine, cysteine, and aspartic proteases, metalloproteases, and aminopeptidases. The concentrations tested were adjusted to have a final EDTA concentration between 0.2 to 2 mM to avoid inhibition of bacterial growth.

2.3. Genomic DNA isolation

S. xylosus C2a gDNA was prepared from overnight cultures grown in Brain Heart Infusion (BHI) broth (Difco Laboratories) with shaking at 37 °C. 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 sulphate and treated with RNase A. Following extraction with phenol-chloroform-isoamyl alcohol (25/24/1) and chloroform, gDNA was precipitated with ethanol and resuspended in Tris–HCl (10 mM; pH 8.5). DNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

2.4. RNA extraction and purification

S. xylosus was grown to mid-log phase and 15 mL of culture was centrifuged. The cell pellet was immediately frozen in liquid nitrogen to stabilize the bacterial RNA and stored at −80 °C. For RNA extraction, the cell pellet was thawed on ice and resuspended in 500 μL of ice-cold Tris-EDTA buffer. The sample was transferred to tubes containing 600 mg of zirconia-silica beads (0.1 mm diameter, BioSpec Products, Bartlesville, OK, USA), 50 μL of sodium dodecyl sulphate (10%), 3.5 μL of β-mercaptoethanol, and 500 μL of acid phenol. Cells were disrupted by two cycles of 60 s each at 6 m/s by using a FastPrep (MP Biomedicals, Illkirch-Graffenstaden, France). After the addition of 200 μL of chloroform and centrifugation, the aqueous phase was collected and purified with the Nucleospin RNA II kit (Macherey Nagel, Hoerdt, France) according to the manufacturer's instructions. A supplementary treatment was performed with Turbo DNAse (Ambion, Austin, TX, USA) to remove residual DNA. The absence of genomic DNA contamination was verified by PCR targeting the *rpoB* gene. Total RNA isolated was quantified using a NanoDrop 1000. The RNA was stored at −80 °C.

2.5. Reverse transcription and quantitative PCR

RNA isolated from three independent biological replicates for each condition or each strain was reverse transcribed to cDNA with SuperScript Reverse Transcriptase according to the manufacturer's

Table 1
Strains and plasmids used in this study.

	Name	Relevant characteristics	Source/Reference
S. xylosus	C2a	Derived from the type strain DSM20267 cured of its endogenous plasmid pSX267	Götz et al., 1983 LN554884
	C2aΔ561	Isogenic mutant of C2a deleted of the SXYL_00561 gene	This work
E. coli	Top 10	Competent strain for plasmid transformation	Invitrogen
Plasmids	pBT2	Temperature-sensitive *E. coli–Staphylococcus* shuttle vector. AmR (*E. coli*) CmR (*Staphylococcus*)	Brückner (1997)
	pSXΔ561	pBT2, SXYL_00561 [13-317]: *ermB* shuttle vector	This work
	pEC4	pBluescript KS + derivative. Source of *ermB* gene (EmR). AmR	Brückner (1997)