Vancomycin heteroresistance and biofilm formation in *Staphylococcus epidermidis* from food

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Staphylococcus epidermidis CNBL 7032 is a heteroresistant strain, with subpopulations resistant to vancomycin concentrations up to 32 mg l⁻¹, which was isolated from cured pork meat. The mechanisms of glycopeptide resistance in this strain were investigated in this study. *S. epidermidis* CNBL 7032 does not harbour enterococcal transmissible vancomycin-resistance genes. Transmission electron microscopy revealed that resistant subpopulations have a thicker cell wall, and that the increase in cell wall thickness is proportional to vancomycin concentration in the growth medium. Scanning electron microscopy showed that *S. epidermidis* CNBL 7032 forms a biofilm-like structure when grown in the presence of vancomycin. This food isolate harbours the gene *atlE*, coding for an autolysin with an adhesive function, which is involved in the first phase of biofilm formation. This study has demonstrated an interaction between *atlE* expression, biofilm formation and glycopeptide antibiotic resistance; transcription analysis demonstrated that the expression of *atlE* increased proportionally with the vancomycin concentration in the culture. Insertional inactivation of *atlE* confirmed the role of the *AtlE* autolysin in biofilm and vancomycin resistance.

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INTRODUCTION

Vancomycin resistance in members of the genus *Staphylococcus* was first reported in 1979 in *Staphylococcus epidermidis*, a species belonging to the group of coagulase-negative staphylococci (CNS) (Siebert *et al.*, 1979). Since this report, vancomycin-resistant CNS have been isolated by several authors and in 1997 the isolation of *Staphylococcus aureus* with decreased susceptibility to vancomycin was first described (Hiramatsu *et al.*, 1997). Various studies have demonstrated that vancomycin resistance in *S. aureus* is characterized by a modification in cell wall metabolism involving an increased rate of cell wall turnover and increased production of penicillin-binding proteins (Hanaki *et al.*, 1998; Livermore, 2000; Boyle-Vavra *et al.*, 2001; Cui *et al.*, 2003). Although the mechanisms of vancomycin resistance of clinical isolates of CNS have been investigated, they are still unclear (Biavasco *et al.*, 2000). For staphylococci, the Clinical and Laboratory Standards Institute has defined susceptibility to vancomycin as a minimal inhibitory concentration (MIC) of ≤ 4 mg l⁻¹; intermediate isolates are those for which the MIC is 8–16 mg l⁻¹ and resistant isolates are those for which the

MIC is ≥ 32 mg l⁻¹ (NCCLS, 2000). Moreover, in staphylococci, a phenomenon of heteroresistance has been described, in which a minority subpopulation with increased resistance to vancomycin is present within a single culture. Heteroresistance has been found more frequently than reduced susceptibility in *S. aureus* and this phenomenon has also been detected in clinical isolates of CNS (Hiramatsu *et al.*, 1997; Srinivasan *et al.*, 2002; Van Der Zwet *et al.*, 2002).

CNS are gaining increasing importance, as they cause a large number of clinically relevant infections in hospitalized patients. In particular, *S. epidermidis* has been identified as a cause of nosocomial infections, associated with the pathogenesis of foreign-body infection due to the ability of the bacteria to colonize the polymer surface by forming a biofilm. This is a two-step process involving different genes coding for adhesins and autolysins (Götz, 2002; Heilmann *et al.*, 2003; Vandecasteele *et al.*, 2003; Vuong *et al.*, 2003; Yao *et al.*, 2005; O'Gara, 2007). The most important factor influencing primary attachment in *S. epidermidis* is the autolysin *AtlE*, encoded by the *atlE* gene (Vuong *et al.*, 2003; Qin *et al.*, 2007). The second phase of biofilm formation requires polysaccharide intercellular adhesin (PIA), which is encoded by the *ica* gene locus (O'Gara, 2007; Ziebuhr *et al.*, 1999). Both molecules are involved in the pathogenesis of intravascular catheter-

Abbreviations: CNS, coagulase-negative staphylococci; PIA, polysaccharide intercellular adhesin; RTq-PCR, quantitative reverse transcriptase polymerase chain reaction; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

associated infection in a rat model (Rupp *et al.*, 2001). In addition, a protein termed Aae has been identified in *S. epidermidis* that has both bacteriolytic and adhesive properties (Heilmann *et al.*, 2003).

Since the natural habitat of CNS is the skin and the mucous membrane of animals, these micro-organisms are often present in raw meat and milk and multiply during food fermentation processes. In particular, in meat fermentation CNS play a fundamental role contributing to reduction of nitrate and nitrite, proteolysis and lipolysis, as well as protection against oxidative product damage. Due to these properties, strains of *Staphylococcus xylosus*, *S. carnosus* subsp. *carnosus*, *S. carnosus* subsp. *utilis*, *S. equorum*, *S. warneri* and *S. saprophyticus* are components of starter cultures used in meat fermentation (Bovercid *et al.*, 1999, 2000; Sondergaard & Stahnke, 2002). Other species can also participate in community development in meat fermentation, and in fact *S. epidermidis* and *S. sciuri*, two CNS occasionally involved in human infections, have been isolated from fermented sausages (Vilar *et al.*, 2000).

The purpose of the present study was to examine the mechanisms of vancomycin resistance in *S. epidermidis* CNBL 7032, a biofilm-forming vancomycin-resistant strain isolated from a cured meat product.

METHODS

Bacterial strains and culture conditions. The bacterial strains examined were isolated from 10 samples of fermented sausages and 10 samples of cured meat (bacon and ham) produced in Europe. Vancomycin-insensitive staphylococci were enumerated on Baird-Parker medium (Oxoid) containing 8 mg vancomycin l⁻¹ (Sigma).

Vancomycin-resistant CNS were cultured on brain heart infusion (BHI) agar (Oxoid) at 37 °C for 24 h. Biofilm formation experiments were performed on positively charged nylon membranes (Boehringer Mannheim) in BHI medium in the presence or absence of vancomycin (32 mg l⁻¹) at 37 °C for 18 h. Cell enumeration was achieved on BHI agar plates after detachment by vortexing for 10 min. The data presented are the means of three different experiments. The isolation of strain CNBL 7032 is described in Results.

Identification of strain CNBL 7032. For taxonomic identification, DNA extracted as previously described (Cocconcelli *et al.*, 1995) was used to amplify a region of 16S rRNA using the primers P1 (5'-GCGGCGTGCTAATACATGC-3') and P6 (5'-CTACGGCTA-CCTTGTTACGA-3'). P1 was used to determine the partial 16S rRNA gene sequence. Taxonomic identification and similarity rank (*S*_{ab}) calculations were performed, comparing the 16S rRNA sequences of isolates with the sequences present in the small subunit database (SSU-Prok) of the Ribosomal Database Project (Maidak *et al.*, 2001).

DNA sequencing. For sequence analysis, the 16S rRNA and *atlE* amplicons were purified using the microCLEAN system (Labogen). Nucleotide sequencing was carried out using an Applied Biosystems 373A DNA sequencer using the ABI PRISM BigDye Terminator Cycle Sequencing and Dye Primer Cycle Sequencing kits (Applied Biosystems).

Susceptibility testing. Resistance to vancomycin was assessed using the agar dilution procedure (NCCLS, 2000) on Müller-Hinton agar (Oxoid). Heteroresistance was determined as follows: three colonies from strain CNBL 7032 grown on BHI agar containing 8 mg vancomycin l⁻¹ (Sigma) were tested for stability of resistance. These colonies were subcultured nine times on antibiotic-free BHI agar. Then vancomycin resistance was tested by plating 100 µl of 10-fold dilutions of cell suspension on Müller-Hinton agar containing 0, 4, 8, 16, 24 or 32 mg vancomycin l⁻¹. Plates were incubated at 37 °C for 48 h (Van Der Zwet *et al.*, 2002).

Detection of vancomycin-resistance determinants. The presence of the vancomycin-resistance *vanA* and *vanB* genotypes was investigated using PCR as described by Clark *et al.* (1993). Primers P1 and P6, which amplify a region of 16S rRNA, were used as an internal control in the PCR. PCR was performed in a volume of 50 µl containing 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.5 µM of each primer, 2.5 U Taq polymerase (Promega) and 300 ng DNA (Cocconcelli *et al.*, 1995), using a GeneAmp PCR system 9700 (Applied Biosystems). Cycling conditions were as follows: 10 min at 95 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C, and a final incubation for 10 min at 72 °C. Amplified products were analysed by gel electrophoresis.

Electron microscopy. Samples for transmission electron microscopy (TEM) were prepared as follows. *S. epidermidis* cells, grown in BHI containing 0, 4, 8, 16, 24 or 32 mg vancomycin l⁻¹, were harvested by low-speed centrifugation (3000 g, 5 min). The cells were washed twice in sodium cacodylate buffer (200 mM, pH 7.3), pre-fixed in 2.5 % (w/v) glutaraldehyde and fixed with 1 % (w/v) OsO₄. The samples were embedded in Epon resin (Fluka Chemie) and thin sections were prepared using a Reichert-Jung Ultracut microtome. Sections were stained with 4 % (w/v) uranyl acetate and then with 0.4 % (w/v) lead citrate, and examined with a JEOL JEE 1200 EXII electron microscope at 100 kV. Cell wall thickness was measured using ImageJ 1.30 software, provided by Wayne Rasband (National Institutes of Health, Bethesda, MD, USA). The data were acquired analysing 80 cell images for each sample.

Samples for scanning electron microscopy (SEM) were prepared as follows. Strains were grown in BHI agar medium (Oxoid) in the absence or presence of vancomycin (4, 8, 16, 24 or 32 mg l⁻¹). Colonies were dehydrated in ethanol/water mixtures, with increasing ethanol concentrations (65 %, 75 %, 85 %, 95 % and 100 %). After critical-point drying in a Baltec CPD030 dryer, specimens were mounted on SEM discs, coated with gold, and observed with a scanning electron microscope (XL30 ESEM, Philips).

Zymographic detection of autolysis. Cells were grown in BHI containing 0, 4, 8, 16, 24 or 32 mg vancomycin l⁻¹ at 37 °C for 18 h, centrifuged, washed with sterile water, and resuspended in a solution of 2 % SDS, 0.01 M EDTA, 0.01 M NaCl and 0.01 M Tris/HCl, pH 8.0. The cell suspension was incubated for 5 min at 100 °C and the cells were pelleted by centrifugation for 10 min. The supernatants were used as the crude surface protein preparations. Protein concentration was determined using the Bio-Rad Protein Assay kit, with BSA (Bio-Rad) as the standard. Autolysins in each sample were resolved by electrophoresis in a minigel apparatus (Bio-Rad), using SDS-PAGE gels (12 %) containing heat-killed *Micrococcus luteus* cells (Sigma) as a substrate for the lytic enzymes. Electrophoresis, denaturation and visualization of clear zones in the gel by methylene blue staining were performed as described by Berger-Bachi *et al.* (1992). Equivalence of loading between lanes was assessed by Coomassie blue staining of SDS-PAGE gels run in parallel (Hanaki *et al.*, 1998; Boyle-Vavra *et al.*, 2001). Densitometric analysis of zymograms was performed using ImageJ 1.3 software, provided by Wayne Rasband (National Institutes of Health, Bethesda, MD, USA).

PCR techniques for detection of adhesion and autolysin genes.

DNA was isolated from *S. epidermidis* CNBL 7032 as described previously (Cocconcelli *et al.*, 1995). Primers were synthesized by Primm (Italy). Primers icaAf (5'-GACCTCGAAGTCAATAGAGGT-3') and icaAr (5'-CCCAGTATAACGTTGGATACC-3') (Ziebuhr *et al.*, 1999) were used to amplify the *ica* gene encoding a polysaccharide intercellular adhesin (PIA) involved in the accumulation phase of biofilm formation. To detect the presence of the *aap* gene, coding for the Aap protein, involved in biofilm formation, primers aapf (5'-ATACAACTGGTGCAGATGGTTG-3') and aapr (5'-GTAGCCGTCCAAGTTTTACCAG-3') were used (Vandecasteele *et al.*, 2003). Primers atlEf (5'-CAACTGCTCAACCGAACA-3') and atlEr (5'-CATCGTTTTTCAGCGCTATCA-3') were used to amplify the *atlE* gene, encoding an autolysin AtlE involved in the initial attachment of cells to the polymer surface and in the second phase of biofilm formation (Vandecasteele *et al.*, 2003). To detect the *aae* gene, encoding a protein with bacteriolytic and adhesive properties, primers aaeF (5'-GAGGAGGATTTTAAAGTGC-3') and aaeR (5'-AACATG-ACCATAGTAACC-3') were used (Heilmann *et al.*, 2003). PCR conditions were as reported previously (Ziebuhr *et al.* 1999; Heilmann *et al.*, 2003; Vandecasteele *et al.*, 2003).

Extraction of total RNA and RT-PCR. Total RNA was extracted from stationary-phase (24 h) *S. epidermidis* cultures grown on BHI agar (Oxoid) containing various concentrations (0, 16 and 32 mg l⁻¹) of vancomycin (Sigma), using the RNeasy Mini kit (Qiagen) with an additional step of treatment with RNase-free DNase (Qiagen). The concentration of RNA was determined by measuring the absorbance at 260 nm using an Ultraspec 2100 pro UV-visible spectrophotometer (Amersham Biosciences).

Reverse transcription reactions were performed on total RNA using the Reverse-iT One-Step RT-PCR kit (ABgene), following the supplier's recommendations, in a final volume of 25 µl containing 30 ng total RNA. Reaction mixtures were incubated at 47 °C for 30 min, followed by denaturation at 94 °C for 2 min. cDNA products were amplified as described above (Vandecasteele *et al.*, 2003). PCR products were analysed on 0.8% agarose gels. To control for the residual presence of DNA, RT-PCR was also performed in the absence of reverse transcriptase. The data are reported as the mean value of five RT-PCR experiments.

Quantitative PCR (RTq-PCR). Quantification was performed using a LightCycler apparatus and the FastStart DNA Master^{Plus} SYBR Green I kit (Roche) using 1 µl or 2 µl of cDNA in a final volume of 20 µl. RNA was prepared as described above, and reverse transcription was performed using 200 ng DNA-free RNA, random primer, and the Transcriptor First Strand cDNA Synthesis kit (Roche), following the supplier's recommendations.

Samples were heated at 95 °C for 10 min before cycling for 45 cycles of 95 °C for 10 s, 55 °C for 20 s, and 72 °C for 25 s. A melting curve plotted at the end of each run verified the specificity of the amplification product. The experiment was repeated three times using cDNA from each of two independent RNA preparations.

The absolute quantification compares the amplification of a target transcript in the sample against a standard curve prepared with known concentrations of the target. The standard curves were generated from tenfold serial dilutions of cDNA and quantified by RTq-PCR using the *gyrA* gene as described by Eleaume & Jabbouri (2004). To quantify the expression of the *atlE* gene, primers were designed with respect to the sequences in the GenBank nucleotide sequence database (accession number U71377) using the Primer 3 website (<http://www-genome.wi.mit.edu>) (Rozen & Skaletsky, 2000) (AtlE_RT_F 5'-CCCAAGGTGCTACTTGCTTC-3'; AtlE_RT_R 5'-TGATAGCGCTGAAAACGATG-3').

Construction of a strain disrupted in the *atlE* gene. A strain disrupted in the *atlE* gene (*S. epidermidis* CNBL 7032Δ*atlE*) was constructed by insertional inactivation of the autolysin gene using the non-replicative plasmid pJIR750 (Chen *et al.*, 2005). A 500 bp mutS fragment was amplified using the HindIII- and BamHI-modified primers atlEf (5'-AAGCTTCAACTGCTCAACCGAACA-3') and atlEr (5'-GGATCCCATCGTTTTTCAGCGCTATCA-3') and cloned into the corresponding sites of pJIR750. The resulting plasmid was electroporated into *S. epidermidis* CNBL 7033 as previously described (Augustin & Gotz, 1990), selecting for chloramphenicol-resistant colonies (15 mg l⁻¹). The insertional inactivation of the *atlE* gene was verified by PCR and Southern hybridization.

RESULTS

Isolation and identification of *S. epidermidis* CNBL 7032

We assessed the occurrence of vancomycin-resistant (MIC ≥ 32 mg l⁻¹) and vancomycin-intermediate (MIC between 8 and 16 mg l⁻¹) staphylococci in fermented and cured meat products by plating samples onto Baird–Parker medium containing 8 mg vancomycin l⁻¹. Vancomycin-intermediate CNS were detected in 16 out of 20 different pork meat products produced in Europe, in numbers varying from 2 × 10¹ to 5.2 × 10⁵ c.f.u. g⁻¹. Vancomycin-intermediate strains were differentiated from vancomycin-resistant strains by observing the replication of isolated colonies on Müller–Hinton medium (Oxoid) agar plates in the presence of 8, 16, 24 and 32 mg vancomycin l⁻¹. Of 96 vancomycin-intermediate CNS, a single strain, CNBL 7032, was able to grow at 32 mg vancomycin l⁻¹ and was used for further studies. The taxonomic identification of this strain was achieved by partial sequencing of the 5' region of 16 S rRNA. The *in silico* analysis revealed that strain CNBL 7032 belonged to the species *Staphylococcus epidermidis*.

S. epidermidis CNBL 7032 is a heteroresistant strain

S. epidermidis CNBL 7032 was examined for the presence of the *vanA* and *vanB* transferable enterococcal genotypes. PCR amplification using DNA extracted from CNBL 7032 and specific primer combinations did not yield any DNA fragments, confirming that the resistance to glycopeptide antibiotics in CNS is not due to the acquisition of enterococcal genetic determinants.

The agar dilution method, performed using the NCCLS procedures (NCCLS, 2000), demonstrated that *S. epidermidis* CNBL 7032 was resistant to up to 32 mg vancomycin l⁻¹. Since heteroresistance has been frequently reported among members of the genus *Staphylococcus*, population analysis experiments were performed to assess the presence of this phenomenon in *S. epidermidis* CNBL 7032. Cultures were grown in the absence or presence of vancomycin, and resistant subpopulations were quantified on agar plates containing different concentrations of vancomycin (0, 8 and

32 mg l⁻¹). *S. epidermidis* CNBL 7032 fulfilled the definition of vancomycin heteroresistance, having subpopulations resistant to 8 and 32 mg vancomycin l⁻¹ at a frequency of 10⁻⁴ and 10⁻⁷, respectively.

Vancomycin influences growth and biofilm formation of *S. epidermidis* CNBL 7032

The observation that *S. epidermidis* CNBL 7032 grown in the presence of vancomycin formed cell aggregates prompted us to examine the effect of the glycopeptide antibiotic on cell envelope properties and biofilm formation. SEM examination of cells grown in the presence of vancomycin revealed the presence of leg-like appendages linking cells (Fig. 1). Moreover, SEM analysis revealed biofilm-like structures only when cells were grown in the presence of high concentrations of vancomycin (24 and 32 mg l⁻¹; Fig. 1E, F).

To assess the relationship between biofilm formation and resistance to vancomycin, *S. epidermidis* CNBL 7032 was grown in a biofilm model on positively charged nylon membranes. High cell density was reached when biofilm was formed on nylon membrane, in both the absence (1 × 10⁹ c.f.u. ml⁻¹) and the presence of 32 mg vancomycin l⁻¹ (9.3 × 10⁸ c.f.u. ml⁻¹). Conversely, in broth cultures

without a solid support, vancomycin strongly reduced the growth rate of *S. epidermidis* CNBL 7032: when cells were grown overnight in liquid medium in the presence of vancomycin, an approximately 900-fold lower cell density (3.5 × 10⁵ c.f.u. ml⁻¹) was observed as compared to control cultures (3.2 × 10⁸ c.f.u. ml⁻¹).

Vancomycin resistance in *S. epidermidis* CNBL 7032 involves cell wall thickening

Preparations of *S. epidermidis* CNBL 7032 grown in the presence of different concentrations of vancomycin were subjected to TEM analysis and morphometric studies. The presence of vancomycin did not influence the cell dimensions: both cell size and diameter were similar in cells grown in the absence or presence of increasing quantities of vancomycin. However, as observed in *S. aureus* (Rice, 2006; Cui *et al.*, 2003), analyses of cell walls (Fig. 2) revealed an increased cell wall thickness in *S. epidermidis* CNBL 7032 cells grown in the presence of vancomycin: the mean cell wall thickness of *S. epidermidis* CNBL 7032 grown with 32 mg vancomycin l⁻¹ was 54.18 nm, ~42 % greater than the mean cell wall thickness of the cells grown in the absence of antibiotic (*P* < 0.001) (Fig. 2).

Fig. 1. SEM of *S. epidermidis* CNBL 7032 subpopulations grown in media containing 0 (A), 4 (B), 8 (C), 24 (D) or 32 (E, F) mg vancomycin l⁻¹. The arrows in (F) indicate leg-like appendages connecting some of the cells.

Fig. 2. TEM of *S. epidermidis* CNBL 7032 grown in media containing vancomycin at 0, 4, 8, 16, 24 or 32 mg l⁻¹. The values under each panel indicate the mean ± SD of cell wall thickness (nm). *, $P < 0.001$, with respect to control values.

Autolytic activity and autolysin production

Since vancomycin resistance in *S. aureus* has been correlated with increased cell wall synthesis (Hanaki *et al.*, 1998) and reduced autolytic properties (Sieradzki & Tomasz, 2003), and because the AtlE autolysin has been linked to the ability to create a biofilm, we investigated the autolytic features of *S. epidermidis* CNBL 7032. Zymographic analysis of autolytic activity revealed a similar pattern and intensity of the cell wall peptidoglycan hydrolases in cells grown with different concentrations of vancomycin (Fig. 3). All samples showed a band with an approximate molecular mass of 120 kDa, which corresponds to AtlE autolysin. The bands with molecular masses of 60 kDa and 52 kDa can be attributed to the amidase and

Fig. 3. Zymogram containing heat-killed cells of *M. luteus* showing autolytic activity of *S. epidermidis* CNBL 7032 grown in media containing various concentrations of vancomycin: 0 (A), 8 (B), 16 (C) or 32 (D) mg l⁻¹. See text for description of arrowed bands.

glucosaminidase domains of AtlE autolysin, respectively. In all samples, the degradation products of the two proteins were present, showing molecular masses of 45 kDa and 38 kDa.

Consistent with the zymogram results, no increase of autolysis was observed when cells were grown in the presence of vancomycin.

atlE transcriptional activity is influenced by vancomycin

Since biofilm formation has been related to vancomycin resistance, we analysed the presence of genes coding for proteins involved in biofilm formation. PCR analysis showed that *S. epidermidis* CNBL 7032 does not harbour *icaA*, *aap* or *aae*, three genes involved in biofilm formation. However, PCR amplification and sequencing experiments confirmed that *S. epidermidis* strain CNBL 7032 contains the *atlE* gene, which codes for the 120 kDa autolysin AtlE, a protein involved in biofilm formation (Heilmann *et al.*, 1997; Vandecasteele *et al.*, 2003; Qin *et al.*, 2007; Nunes *et al.*, 2006). To evaluate if the expression of this gene was influenced by the glycopeptide antibiotic and by biofilm formation, total RNA was extracted from stationary-phase cells grown with increasing concentrations of vancomycin and used for RT-PCR experiments. Vancomycin positively influenced the transcriptional activity of *atlE*, since the expression of the gene was proportional to the concentration of antibiotic in the growth medium (Fig. 4).

The results of RT-PCR were confirmed by RTq-PCR. Standard curves were analysed using LightCycler Analysis software and regression quantification gave regression lines with slope -3.078, error of 0.102 and r of -1.00. The expression profile of each transcript calculated versus an external standard curve showed an increase of *atlE*

Fig. 4. RT-PCR analysis of transcriptional activity of the *atlE* gene from *S. epidermidis* cells grown in the presence of different concentrations of vancomycin: the bars indicate the fold increase in expression of *atlE*, compared to the expression level in absence of vancomycin. Data are the means of five different experiments.

expression proportional to the increased concentration of vancomycin in the growth medium (data not shown).

At a vancomycin concentration of 16 mg l⁻¹, a slight increase (13%) in *atlE* expression was observed, as compared with control cells grown in the absence of vancomycin. A sharp, fourfold increase was observed at 32 mg l⁻¹ or when cells were grown in biofilm on a nylon surface.

Construction and characterization of the CNBL 7032Δ*atlE* mutant.

The *atlE* gene was disrupted by insertion to create strain CNBL 7032Δ*atlE*. The correct insertion was confirmed by PCR and Southern hybridization (data not shown). RT-PCR experiments demonstrated that CNBL7032Δ*atlE* did not express the *atlE* gene. Although the growth rates of the parent strain and CNBL7032Δ*atlE* in BHI medium were substantially similar, as observed by Qin *et al.* (2007), the ability to form biofilm on nylon membrane was significantly depressed by *atlE* inactivation.

The agar dilution method showed that *S. epidermidis* CNBL7032Δ*atlE* was susceptible to vancomycin (MIC 4 mg l⁻¹). Moreover the heteroresistance phenomenon observed in the parent strain was not detected in the *atlE* mutant.

DISCUSSION

The use of glycopeptide antibiotics as growth promoters in animals may have favoured the evolution of vancomycin-resistant bacteria in the food chain. Since coagulase-negative bacteria are a dominant population in meat products, the diffusion of vancomycin resistance in *S. epidermidis* has been described only recently (Nunes *et al.*, 2006). The present work aimed to assess the occurrence of vancomycin resistance in food-associated CNS and to study the mechanism of vancomycin resistance in a food

isolate of *S. epidermidis*. Among 96 strains of staphylococci with intermediate vancomycin resistance isolated from fermented and cured meat products, only one strain able to grow at 32 mg vancomycin l⁻¹ was isolated. Our study demonstrated that this strain, *S. epidermidis* CNBL 7032, is heteroresistant, since only a small proportion of the population (10⁻⁷) is resistant to vancomycin concentrations as high as 32 mg l⁻¹. Given that this strain cohabits in meat products with enterococci, and that horizontal transmission of vancomycin-resistance genetic determinants between *Enterococcus* and *Staphylococcus* has been demonstrated (Noble *et al.*, 1992; Showsh *et al.*, 2001; Flannagan *et al.*, 2003), we looked for the presence of enterococcal vancomycin-resistance determinants in *S. epidermidis* CNBL 7032 by PCR, but none of the transmissible glycopeptide-resistance genotypes were detected. Rather, morphometric analysis by TEM revealed that *S. epidermidis* CNBL 7032 modifies the thickness of its cell walls as a function of the vancomycin concentration, a phenomenon that has been well described as a resistance mechanism in clinical isolates of *S. aureus* and *S. epidermidis* (Nunes *et al.*, 2006). Separate studies from different laboratories have shown that the mechanism of resistance to glycopeptide antibiotics in coagulase-positive staphylococci is based on a thickened cell wall with decreased cross-linking and increased cell wall metabolism (Hanaki *et al.*, 1998; Geisel *et al.*, 1999; Reipert *et al.*, 2003). Our study demonstrated that the mechanism of vancomycin resistance in *S. epidermidis* isolated from food appears to depend upon modifications in cellular metabolism resulting in an increase in cell wall thickness.

Furthermore, our data suggest that other mechanisms are involved in vancomycin resistance in *S. epidermidis* CNBL 7032, since this strain can grow at higher cell density in the presence of 32 mg vancomycin l⁻¹ on solid media or in a liquid medium in the presence of a solid support that allows the formation of a biofilm. Genes involved in biofilm formation have been studied in *S. epidermidis*, since this physiological property has been associated with virulence (Heilmann *et al.*, 1997; Von Eiff *et al.*, 2002; Vandecasteele *et al.*, 2003; Vuong *et al.*, 2003) and antibiotic resistance (Mønsson *et al.*, 2002). In particular, the *ica* operon, *aap*, *aae* and *atlE* genes, coding respectively for PIA adhesin, Aap protein, Aae surface-associated protein and AtlE cell wall autolysin, have been related to biofilm formation. Biofilm-like structures were observed by electron microscopy when *S. epidermidis* CNBL 7032 was grown in the presence of vancomycin (Fig. 1), as was observed in strains of the same species that formed biofilm on biomedical materials and correlated with the *ica* gene (Kodjikian *et al.*, 2003a, b).

We also investigated the interaction between genes involved in biofilm formation and glycopeptide antibiotic resistance. Mønsson *et al.* (2002) reported that vancomycin has a reduced activity toward *S. epidermidis* growing in biofilms. In addition, Jefferson *et al.* (2004) demonstrated that a glycopeptide-insensitivity-associated gene implicated

in biofilm formation in staphylococci is involved in the regulation of *icaR*. Our PCR analyses demonstrated the absence of the *ica* operon, coding for the PIA adhesin, and the autolysin *aae* gene, two genes which have been previously reported to be involved in the second phase of biofilm formation (Rupp *et al.*, 2001; Heilmann *et al.*, 2003). Conversely, *S. epidermidis* CNBL 7032 contained the gene *atlE* coding for the AtlE autolysin, which has an adhesive function that is involved in the first phase of biofilm formation, as recently described by Qin *et al.* (2007).

Our present data suggest that the vancomycin resistance of *S. epidermidis* CNBL 7032 is influenced by biofilm formation, cell wall thickness and *atlE* gene expression. In the presence of vancomycin, *S. epidermidis* CNBL 7032 showed a significant increase (about 42%) in cell wall thickness, suggesting that cells grown in the presence of high concentrations of vancomycin increase their production of AtlE. Quantitative RT-PCR experiments demonstrated that expression of the *atlE* gene increased proportionally as the concentration of antibiotic in the medium increased. The insertional inactivation of the *atlE* gene confirmed the role of AtlE in *S. epidermidis* CNBL 7032 in vancomycin resistance.

Since Sieradzki & Tomasz (2003) have demonstrated that vancomycin resistance is related to modification of autolysis activity in *S. aureus*, we assessed the autolytic activity of vancomycin-resistant *S. epidermidis* CNBL 7032 subpopulations. Zymogram analysis of autolytic enzymes showed a similar proportion between total cell wall proteins and autolysins in cells grown at different vancomycin concentrations.

Food has been demonstrated to be a potential reservoir of vancomycin-resistant bacteria, including members of the genus *Enterococcus*. Thus, the isolation of vancomycin-insensitive staphylococci from cured meat products raises questions about the safety of antibiotic-resistant CNS strains in food chains and should stimulate further studies to assess the risks associated with consumption of food containing relevant amounts of antibiotic-resistant bacteria.

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