INTRODUCTION

The bacterial phenomenon of quorum sensing enables bacteria to communicate and thereby coordinate the expression of specific target genes in response to the population density. In a minimum of 30 Gram-negative bacterial species, quorum sensing is mediated by small diffusible signal molecules, N-acylated derivatives of L-homoserine lactone, which may differ in the length and substitution of their respective acyl side chains. N-Acyl-L-homoserine lactone (AHL) molecules with N-acyl side chains ranging from 4 to 14 carbons and with an oxo-, hydroxy- or no substituent at the C3 position have been identified [for recent reviews see Whitehead et al. (2001), Fuqua et al. (2001) and Miller & Bassler (2001)]. One of the best characterized quorum sensing systems is present in the marine bacterium Vibrio fischeri, in which LuxI synthesizes N-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C6-HSL). It is believed that the signal molecule 3-oxo-C6-HSL at a certain threshold concentration binds to a regulatory protein, LuxR, and thereby renders it active. LuxR hereafter activates transcription of the luxICDABEG operon. As a consequence, the bacterial culture expresses a bioluminescent phenotype when it colonizes the light organs of certain fish and squids and a non-bioluminescent phenotype when the bacteria live in a planktonic state in sea water [(for reviews see Sitnikov et al. (1995) and Dunlap (1999)]. This is one of the few known examples of quorum-sensing-regulated gene expression in relation to a symbiotic relationship. In most other cases, quorum sensing is related to pathogenicity traits such as conjugal transfer of the Ti plasmids from Agrobacterium tumefaciens (Piper et al., 1993; Zhang et al., 1993), the production of extracellular cell-wall-degrading enzymes in Erwinia carotovora (Jones et al., 1993; Pirhonen et al., 1993) and the induction of virulence factors in Pseudomonas aeruginosa (Jones et al., 1993; Pearson et al., 1994; Winson et al., 1995; Latifi et al., 1995). In addition,
there is growing evidence that the ability to form surface-associated, structured and co-operative consortia (referred to as biofilms) is also controlled by quorum sensing (Davies et al., 1998; Eberl et al., 1996; Huber et al., 2001). These properties all play a significant role in bacterial pathogenesis and are common causes of persistent infections (Costerton et al., 1999).

At the molecular level, the majority of the quorum sensing regulatory R-proteins described to date work as transcriptional activators; however, few R-proteins have been suggested to work as repressors. The most thoroughly described R-protein functioning as a repressor is EsAR of Pantoea stewartii (formerly Erwinia stewartii). It has been suggested that EsAR represses the synthesis of extracellular polysaccharide at low cell density, and that the presence of its cognate signal molecule in micromolar amounts results in derepression (Beck von Bodman & Farrand, 1995; Beck von Bodman et al., 1998; Minogue et al., 2002). Other examples of R-proteins that have been proposed to function as repressors include SpnR of Serratia marcescens and YpsP of Yersinia pseudotuberculosis (Atkinson et al., 1999; Horng et al., 2002).

The AHL-producing phenotype is widespread among members of the Enterobacteriaceae (e.g. Serratia liquefaciens, Enterobacter agglomerans, Erwinia carotovora, Hafnia alvei and Rahmella aquatilis) (Eberl et al., 1996; Swift et al., 1993, 1999). It has been demonstrated that the ability to produce ACHs is a common feature among Enterobacteriaceae isolated from food products such as chilled, vacuum-packed meat and cold-smoked salmon (Gram et al., 1999; Ravn et al., 2001). These psychrophilic members of the Enterobacteriaceae are frequently encountered in the microflora of spoiling food products such as milk, cream, fish and minced meat, and are in some products involved in spoilage process (Borch et al., 1996; Lindberg et al., 1998).

The activities of hydrolytic enzymes produced by bacteria have been linked to the spoilage of raw food products. Proteolytic and lipolytic activities of psychrophilic Gram-negative bacteria are regarded as a cause of deterioration of milk and other dairy products (Burger et al., 2000; Tan & Miller, 1992). Also, the soft-rot spoilage of vegetables and fruits is often caused by the pectinolytic activity of pseudomonads or Enterobacteriaceae (mostly Erwinia spp.) (Chatterjee et al., 1994; Liao, 1989). Interestingly, the pectinase of Erwinia carotovora is regulated by acylated homoserine lactone (Pirhonen et al., 1993), suggesting that rot is controlled by a quorum-sensing mechanism. In S. marcescens and S. liquefaciens MG1, a functional lipB operon is required for the secretion of several unrelated and potentially food-quality-relevant proteins such as the lipase LipA, the metalloprotease PrtA and the surface-layer protein (S-layer) SiaA (Akatsuka et al., 1997; Kawai et al., 1998; Riedel et al., 2001). The LipB protein translocation system is a type I secretion apparatus belonging to the superfAMILY OF ATP-BINDING CASSETTE TRANSPORTERS (ABC) that is dedicated to proteins lacking an N-terminal signal peptide [(for a review see Binet et al. (1997)]. The structural organization and sequence conservation of the genes encoding the ABC protein translocation systems is highly conserved among the Gram-negative bacteria (Binet et al., 1997). The lipB operon of S. marcescens consists of three genes, lipBCD, encoding an inner-membrane ATPase (the ABC protein), a membrane fusion protein and an outer-membrane polypeptide, respectively (Akatsuka et al., 1995, 1997). S. liquefaciens MG1 has been demonstrated to be under the transcriptional control of N-butyryl-L-homoserine lactone (C4-HSL), which renders the production of extracellular protease activity indirectly under the control of quorum sensing (Riedel et al., 2001). In the present study, we have analysed quorum sensing regulation of protein expression in S. proteamaculans and have demonstrated that the activities of several exoenzymes are affected by 3-oxo-C6-HSL. This is the first report linking AHL-type signal molecules and chitinolytic activity in Serratia spp. In addition, the activities of food-quality-relevant phenotypes (i.e. expression of lipase and protease) are affected by 3-oxo-C6-HSL, which leads us to conclude that quorum sensing is involved in the control of food-spoilage processes.

METHODS

Strains and bacterial growth conditions. The strains used in this study are listed in Table 1. S. proteamaculans, Chromobacterium violaceum and A. tumefaciens were grown at 25°C. Escherichia coli was grown at 37°C. All strains were grown in Luria–Bertani (LB) growth medium (Bertani, 1951) containing 4 g NaCl l\(^{-1}\) instead of the normal 10 g NaCl l\(^{-1}\) or AB minimal medium (Clark & Maaloe, 1967) containing 0-5 % (w/v) glucose and 0-5 % (w/v) Casamino acids. Antibiotics were added in the following amounts (m\(^{-1}\)) if necessary: 100 µg ampicillin, 50 µg kanamycin, 10 µg chloramphenicol, 5 µg tetracycline and 50 µg streptomycin for Escherichia coli; 50 µg gentamicin for A. tumefaciens; 50 µg kanamycin for C. violaceum; 25 µg nalidixic acid, 25 µg kanamycin and 15 µg streptomycin for S. proteamaculans.

Extraction of chromosomal DNA. Chromosomal DNA was extracted as described previously (Givskov et al., 1995).

PCR amplification of 16S rDNA. The oligonucleotides used in this study are listed in Table 1. The amplification of the 16S rDNA was performed using the Expand High Fidelity PCR System according to manufacturer’s instructions (Boehringer Mannheim). Using approximately 2 µg chromosomal DNA, primers 9F and 1512R and 1-5 mM MgCl\(_2\), PCR was performed on a Biometra Thermocycler. The thermal profile consisted of an initial denaturation step of 5 min at 94°C followed by 30 cycles of 15 s at 94°C, 30 s at 55°C and 90 s at 72°C, followed by a final step of 5 min at 72°C. The PCR product was purified using a GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech).

DNA manipulations. Standard techniques for DNA manipulations were used (Sambrook et al., 1989). pet and pet-derived vectors were propagated in Escherichia coli CC118/pir. For construction of the pet-derived luxAB-Sm’ vector pAC25, the 3-2 kb NotI fragment of pUTtcuxAB (spanning the promoterless luxAB genes) was ligated into the single NotI site of pUTSm. The orientation of the luxAB cassette was investigated by digesting pAC25 with Sphi. A 3-2 kb fragment indicated that the luxAB cassette had been inserted in the correct orientation (data not shown). For construction of pAC26, chromosomal DNA of S. proteamaculans AC1 was digested with...
BglII and ligated into the BamHI site of pLOW1. Kanamycin-resistant *Escherichia coli* MT102 clones containing pAC26 carried a DNA fragment of approximately 12 kb. For construction of pAC27, chromosomal DNA of *S. proteamaculans* AC2 was digested with BglII and ligated into the BamHI site of pLOW1. Bioluminescent *Escherichia coli* MT102 clones containing pAC27 carried a DNA fragment of approximately 6 kb.

**Table 1.** Strains, plasmids and oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid/oligonucleotide</th>
<th>Characteristic(s)</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Agrobacterium tumefaciens</em> NT1</td>
<td>Ti plasmidless; prototrophic</td>
<td>Watson et al. (1975); Cha et al. (1998)</td>
</tr>
<tr>
<td><em>Chromobacterium violaceum</em> CV026</td>
<td>Km’, Hg’; double mini-Tn5 mutant derived from <em>C. violaceum</em> ATCC 31532; autoinducer sensor</td>
<td>Winson et al. (1994); McClean et al. (1997)</td>
</tr>
<tr>
<td><em>Escherichia coli</em> MT102</td>
<td>Sm’; restriction-negative mutant derived from MC1000</td>
<td>M. T. Hansen (unpublished data)</td>
</tr>
<tr>
<td><em>Escherichia coli</em> HB101</td>
<td>Sm’; recA thi pro leu hisDM</td>
<td>Kessler et al. (1992)</td>
</tr>
<tr>
<td><em>Escherichia coli</em> CC118/pir</td>
<td>Δ(ara–leu) araD ΔluxX74 galE galK phoA thi-1 rpsE rpoB argE(Am) recA1</td>
<td>Herrera et al. (1990)</td>
</tr>
<tr>
<td><em>Serratia proteamaculans</em> B5a</td>
<td>Wild-type</td>
<td>Gram et al. (1999); Paludan-Müller et al. (1998)</td>
</tr>
<tr>
<td><em>Serratia proteamaculans</em> B5aN</td>
<td>Nal’; wild-type derivative of B5a</td>
<td>This study</td>
</tr>
<tr>
<td><em>Serratia proteamaculans</em> AC1</td>
<td>Nal’, Km’; mini-Tn5 insertion in <em>sprI</em></td>
<td>This study</td>
</tr>
<tr>
<td><em>Serratia proteamaculans</em> AC2</td>
<td>Nal’, Km’, Sm’; ‘luxAB; AC1 derivative containing mini-Tn5 insertions in <em>sprI</em> and <em>lipB</em></td>
<td>This study</td>
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<tr>
<td><strong>Plasmid</strong></td>
<td></td>
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<tr>
<td>pDZLR4</td>
<td>Gm’; traR traG::lacZ</td>
<td>Cha et al. (1998)</td>
</tr>
<tr>
<td>pSB403</td>
<td>Tc’; luxR luxI::luxCDABE</td>
<td>Winson et al. (1998)</td>
</tr>
<tr>
<td>pLOW1</td>
<td>Cam’; pACYC184-derived cloning vector</td>
<td>Hansen et al. (1997)</td>
</tr>
<tr>
<td>pRK600</td>
<td>Cam’; ColE1oriV RP4oriT RP4tra+</td>
<td>Kessler et al. (1992)</td>
</tr>
<tr>
<td>pUTKm</td>
<td>Ap’, Km’; delivery vector for mini-Tn5 Km’</td>
<td>de Lorenzo et al. (1990)</td>
</tr>
<tr>
<td>pUTsm</td>
<td>Ap’, Sm’; delivery vector for mini-Tn5 Sm’</td>
<td>de Lorenzo et al. (1990)</td>
</tr>
<tr>
<td>pUTcLuxAB</td>
<td>Ap’, Tc’; delivery vector for mini-Tn5 ‘luxAB-Tc’. NotI sites flank the promoterless 3-2 kb luxAB reporter genes</td>
<td>de Lorenzo et al. (1990)</td>
</tr>
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<td>pAC25</td>
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<td>This study</td>
</tr>
<tr>
<td>pAC26</td>
<td>Cam’, Km’; pLOW1 containing approx. 12 kb of <em>S. proteamaculans</em> AC1 DNA</td>
<td>This study</td>
</tr>
<tr>
<td>pAC27</td>
<td>Cam’, Sm’; pLOW1 containing approx. 6 kb of <em>S. proteamaculans</em> AC2 DNA</td>
<td>This study</td>
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<td><strong>Oligonucleotide</strong></td>
<td></td>
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<tr>
<td>9F</td>
<td>5’-GAGTTTGATCCTGGCTCAG</td>
<td>Snaidr et al. (1997)</td>
</tr>
<tr>
<td>1512R</td>
<td>5’-ACGCGGTACCTGGTTACGACCTT</td>
<td>Lane (1991)</td>
</tr>
<tr>
<td>AC14a</td>
<td>5’-GAATACCATCCGCTCCTAGG</td>
<td>This study</td>
</tr>
<tr>
<td>AC14b</td>
<td>5’-CCATGAGCCGTATGGTTATTC</td>
<td>This study</td>
</tr>
<tr>
<td>AC24</td>
<td>5’-CAGTTGGACACACATATTCC</td>
<td>This study</td>
</tr>
<tr>
<td>AC27</td>
<td>5’-GCTTAAAGGCTTAAAGGATAT</td>
<td>This study</td>
</tr>
<tr>
<td>AC30/i-end</td>
<td>5’-CAGATCTGATCAGAGACACAG</td>
<td>This study</td>
</tr>
<tr>
<td>AC31/o-end</td>
<td>5’-CACTTCCTGTATAAGATCAG</td>
<td>This study</td>
</tr>
<tr>
<td>AC40a</td>
<td>5’-CATATGTTCTCACTAACCAGA</td>
<td>This study</td>
</tr>
<tr>
<td>AC50/luxA</td>
<td>5’-CAAGCGACGTTCCATTACAGT</td>
<td>This study</td>
</tr>
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</table>

**Construction of an AHL-negative mutant of *S. proteamaculans* B5aN.** A bank of random insertion mutants was made using the mini-Tn5 transposon delivery system described by Herrero et al. (1990). In brief, *Escherichia coli* CC118/pir harbouring pUTKm was used as donor, *Escherichia coli* HB101 harbouring pRK600 was used as helper and *S. proteamaculans* B5aN was used as recipient. The three strains were mixed in a ratio of 1:1:5 (donor:helper:recipient) and incubated on an LB agar plate for 6 h at 30°C. The mutant bank was plated onto selective LB plates (containing naldixic acid and kanamycin) and incubated for 12 h. To screen for 3-oxo-C6-HSL-negative mutants, the resulting bank of insertion mutants was replica-plated onto indicator LB plates containing...
approximately 1 % (v/v) outgrown culture of the monitor bacterium C. violaceum CV026. Putative AHL-negative clones were unable to induce violacein production after 12 h incubation.

**Construction of quorum sensing target gene mutants of S. proteamaculans AC1.** A bank of secondary transposon insertion mutants was made using the mini-Tn5 transposon delivery system described by Herrero et al. (1990). *Escherichia coli* CC118/pir harbouring pAC25 (‘luxAB-Sm’) was used as donor, *Escherichia coli* HB101 harbouing pRK600 was used as helper and *S. proteamaculans* AC1 was used as recipient. The secondary mutant bank was plated onto selective LB plates (containing kanamycin and streptomycin) and incubated for 12 h. To screen for potential quorum-sensing-regulated genes, the resulting bank of double mutants was replica-plated onto selective LB plates and indicator LB plates containing 100 nM of 3-oxo-C6-HSL (Sigma-Aldrich; CAS no. 143537-62-6) and incubated for 12 h. Bioluminescent clones are expected to have the promoterless luxAB cassette inserted as a transcriptional fusion. The activities of bioluminescent transconjugants were compared using a Hamamatsu charge-coupled device camera connected via a Hamamatsu M4314 controller to a Hamamatsu Argus-50 image processor (Hamamatsu Photonics). Ten mutants with elevated bioluminescence expression on LB plates containing 3-oxo-C6-HSL were selected for further analysis.

**Screening for protease-deficient secondary mutants.** The collection of secondary mutants with insertions in potential quorum sensing target genes was screened for proteolytic activity on LB plates containing 0.5 % (w/v) skim milk powder. One mutant, *S. proteamaculans* AC2, was unable to make clearing zones on the agar after 24 h incubation.

**DNA nucleotide sequencing.** The oligonucleotides used in this study are listed in Table 1. Sequencing was performed at a commercial sequencing facility (K. J. Ross-Petersen AS; http://www.ross.dk/). PCR primers 9F and 1512R were used as sequencing primers for the 16S rDNA. Primers AC14a, AC14b, AC24, AC27, AC30/i-end, AC31/o-end and AC40a were used as sequencing primers for the 16S rDNA. Primers AC14a, AC14b, AC24, AC27, AC30/i-end, AC31/o-end and AC40a were used as sequencing primers for the sprl and sprR genes present on pAC26. The sequencing primer AC50/luxA was used for sequencing of the upstream DNA of the ‘luxAB-Sm’ cassette present on pAC27.

**DNA sequence analysis.** BLASTN and BLASTX homology searches for the DNA sequences in the non-redundant sequence databases were performed via the worldwide web at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple-sequence alignment was performed using the CLUSTAL W algorithm at Network Protein Sequence Analysis (http://pbil.ibcp.fr/npsa_clustalw.html).

**Southern blot.** Analyses of Southern blots were performed as described previously (Givskov et al., 1995). Chromosomal DNA of *S. proteamaculans* AC1 was digested with *KpnI* and *PstI*, and chromosomal DNA of *S. proteamaculans* AC2 was digested with *BglII* and *XhoI*. A DIG-labelled kanamycin-resistance gene and luxAB were used as probes against *S. proteamaculans* AC1 and *S. proteamaculans* AC2, respectively.

**SDS-PAGE.** Standard SDS-PAGE was performed as described by Laemmli (1970). Proteins were visualized by silver staining according to Blum et al. (1987).

**Two-dimensional PAGE analysis of total intracellular proteins.** *S. proteamaculans* B5aN and *S. proteamaculans* AC1 were grown in AB minimal medium until an OD<sub>650</sub> value of approximately 10 was reached. Culture supernatants were harvested by centrifugation at 10 000 g for 1 h. To get a clearly focused protein pattern, a phenol extraction on bacterial extracts was performed as described previously (Hanna et al., 2000). In a 2 ml screw-cap tube, 1 ml aliquots of the extracts and 1 ml of phenol were mixed thoroughly and incubated for 10 min at 70°C. The sample was cooled on ice for 5 min and the phases were separated by centrifugation for 10 min at 5000 g. The top aqueous phase was discarded and 1 ml distilled water was added. After vortexing and incubation for 10 min at 70°C, the sample was cooled and the phases were separated as before. The aqueous phase was discarded and the proteins were precipitated by adding 1 ml ice-cold acetone. The sample was pelleted by centrifugation for 20 min at 10 000 g, and the pellet was washed by adding 1 ml ice-cold acetone. After a final centrifugation step (20 min at 10 000 g), the supernatant was poured off and the pellet was air-dried for 30 min. The protein precipitate from the phenol extraction was solubilized in 1 ml lysis buffer [9 M urea, 2 % (w/v) CHAPS, 0.8 % (w/v) Phosmalyte pH 3–10 (Amersham Pharmacia Biotech), 1 % (w/v) DTT and 5 mM Pefabloc (Merck)]. For the isoelectric focusing, 8 μl of the samples were mixed with 342 μl of rehydration solution [8 M urea, 2 % (w/v) CHAPS, 15 mM DTT and 0.5 % (w/v) IPG-buffer pH 3–10 (Amersham Pharmacia Biotech)] resulting in a final protein amount of 110 μg per sample. The isoelectric focusing was performed by using immobilized pH gradient (IPG) strips (Amersham Pharmacia Biotech) as described elsewhere (Görg et al., 2000). The IPG strips (18 cm, pH 3–10) were rehydrated overnight at 30 V and focused for 3 h at 8000 V at 20°C under mineral oil. They were then incubated for 10 min in each case in equilibration buffer I [6 M urea, 30 % (w/v) glycerol, 2 % (w/v) SDS and 1 % (w/v) DTT in 0.5 M Tris/ HCl buffer, pH 8.3] followed by equilibration buffer II [6 M urea, 30 % (w/v) glycerol, 2 % (w/v) SDS and 4 % (w/v) iodoacetamide in 0.5 M Tris/HCl buffer, pH 8.8]. After the equilibration step, the strips were transferred to 22 x 22 cm 12-5 % SDS-PAGE gels for the second dimension. Electrophoresis was performed at 150 V and 150 mA at 15°C for approximately 18 h as described previously (Görg et al., 2000).

Protein spots were visualized by silver staining as described elsewhere (Blum et al., 1987). The gels were scanned with a densitometric ImageScanner (Amersham Pharmacia Biotech) and the raw images were processed using the software IMAGE MASTER 2-D ELITE, version 3.0 (Amersham Pharmacia Biotech). Following spot editing and background subtraction, the protein patterns were matched to each other by visual inspection.

**Measurements of bioluminescence.** Bioluminescence was quantified in a Bio-Orbit 1253 luminometer as described previously (Givskov et al., 1998).

**Extraction of culture supernatants and analytical TLC.** AHL extractions and TLC were performed as described previously (Ravn et al., 2001). For the detection of AHLs, three different biomonitors were used as described previously, *C. violaceum* CV026 (McClellan et al., 1997), *A. tumefaciens* NT1 harbouring pDZLR4 (Cha et al., 1998) and *Escherichia coli* MT102 harbouring pSB403 (Winson et al., 1998).

**Enzymic assays.** *S. proteamaculans* B5aN, *S. proteamaculans* AC1 and *S. proteamaculans* AC2 were grown in LB medium until an OD<sub>650</sub> value of approximately 10 was reached. Culture supernatants were harvested and filtered-stereilized prior to enzymic examinations. Chitinolytic activity was investigated on carboxymethyl-chitin-remaol brilliant violet (CM-chitin-RBV) by incubating 450 μl of a substrate mixture with 150 μl culture supernatant. The substrate mixture contained 1 volume of 0.2 % (w/v) CM-chitin-RBV and 2 volumes of succinate/NaOH buffer [100 mM] (pH 6.0). The mixture was incubated at 37°C for 6 h. The enzymic reaction was stopped by adding 200 μl of 2 M HCl to the mixture and incubating...
at 0°C for 1 h. Prior to spectroscopic measurement, the mixture was centrifuged for 10 min at 15000 g. The chitinolytic activity was quantified by the determination of the OD540 value. Lipolytic activity was investigated as described previously (Riedel et al., 2001). One millilitre of a reaction mixture containing 100 μl bacterial supernatant and 900 μl substrate mixture was incubated for 1 h at room temperature. The substrate mixture contained 1 volume of 0.3% (w/v) p-nitrophenyl palmitate in 2-propanol and 9 volumes of 0.2% (w/v) sodium deoxycholate and 0.1% (w/v) guammi arabic in 50 mM sodium phosphate buffer (pH 8.0). The reaction mixture was centrifuged at 15000 g for 5 min, and the enzymic reaction was terminated by adding 1-0 ml of 1 M Na2CO3 to the supernatant prior to spectroscopic measurement. The lipolytic activity was quantified by determination of the OD440 value. Proteolytic activity was investigated as described previously (Ayora & Gotz, 1994). An aliquot (150 μl) of filter-sterilized culture supernatant was incubated with 250 μl substrate for 6 h at 37°C. The substrate contained 1% (w/v) azoalbumin (pH 7-5). The enzymic reaction was terminated by adding 1-2 ml of 10% (w/v) trichloroacetic acid. The mixture was incubated for 15 min at room temperature and then centrifuged for 10 min at 15000 g. Prior to spectroscopic measurement, 600 μl supernatant was rescued and mixed with 750 μl of 1 M NaOH. The proteolytic activity was quantified by the determination of the OD440 value. Specific enzymic activities were determined as the enzymic activity per density unit (OD450) of the growth culture.

Zymograms. Chitinases were analysed by SDS-PAGE (15% polyacrylamide). The substrate CM-chitin-RBV was incorporated into the gel matrix to a final concentration of 0.05% (w/v). After electrophoresis, the proteins were renatured by washing the gel twice in a mixture containing 50 mM Tris/HCl (pH 6-0) and 25% (v/v) 2-propanol for 15 min at room temperature. After renaturation, the zymogram was incubated for 24 h at 30°C in 50 mM Tris/HCl (pH 6-0). Enzymic activity was detected as clear zones on a violet background. To improve contrast, the colours of the picture were inverted. Exolipase (or esterase) was analysed by SDS-PAGE (12% polyacrylamide). After electrophoresis, the proteins were renatured by washing the gel twice in a mixture containing 50 mM Tris/HCl (pH 7-5) and 25% (v/v) 2-propanol for 15 min at room temperature and once in 50 mM Tris/HCl (pH 7-5). The renatured gels were overlaid with a fluorescent substrate, 0-01 M methylumbelliferyl butyrate dissolved in N,N-dimethylformamide (Sigma), and incubated for 30 min at 30°C. The enzymes could be detected with UV light (360 nm) as blue fluorescent bands. Exoproteases were analysed by SDS-PAGE of culture supernatants with 0.2% azoalbumin incorporated in the gel matrix (12% polyacrylamide). After electrophoresis, the proteins were renatured by washing the gel twice in a mixture containing 50 mM Tris/HCl (pH 7-5) and 25% (v/v) 2-propanol for 15 min at room temperature. After renaturation, the zymogram was incubated for 4 h in 50 mM Tris/HCl (pH 7-5). Prior to detection of the proteins, the gel was washed in 1 M NaOH for 5 min. Protease activity could be detected as colourless zones in an orange background.

Food spoilage. Samples of pasteurized and homogenized cows milk containing 3-4% protein, 1.5% lipid and 4-8% carbohydrates were inoculated with approximately 1×10^6 c.f.u. ml^-1 of S. proteamaculans B5aN, S. proteamaculans AC1 or the S. proteamaculans AC2. Samples inoculated with S. proteamaculans AC1 or S. proteamaculans AC2 were grown in the absence and presence of 200 nM of 3-oxo-C6-HSL. The samples were incubated at room temperature for 18 h.

RESULTS AND DISCUSSION

S. proteamaculans B5a was isolated from spoiled cold-smoked salmon and initially classified as S. liquefaciens B5a (Gram et al., 1999; Paludan-Müller et al., 1998). A phylogenetic analysis based on the 16s rDNA sequence of B5a indicated a more relevant position as S. proteamaculans (GenBank accession no. AY040208; data not shown). S. proteamaculans B5aN is a nalidixic-acid-resistant derivative of S. proteamaculans B5a.

Construction of an AHL-deficient mutant

Using TLC on extracted, spent media, we have previously shown that the major AHL signal molecule produced by S. proteamaculans B5a is 3-oxo-C6-HSL (Gram et al., 1999). Interestingly, the close relatives S. marcescens and S. liquefaciens MG1 produce C4-HSL as their main products (Eberl et al., 1996). S. liquefaciens MG1 produces C4-HSL and N-hexanoyl-L-homoserine lactone in a ratio of 10:1 (Eberl et al., 1996).

An AHL-deficient S. proteamaculans mutant (denoted AC1) was constructed by random transposon mutagenesis as described in Methods. A Southern blot analysis confirmed that a single copy of the transposable element had been integrated into the chromosome of this mutant (data not shown). DNA sequence analysis on the flanking regions of the kanamycin insertion in S. proteamaculans AC1 indicated the insertion to 220 bases downstream from the translation start in a gene belonging to the family of luxI homologous AHL synthases (Fig. 1a). Extracted, spent medium from the sprI mutant (AC1) was subjected to a TLC-based analysis and developed using CviR-, LuxR- and TraR-based AHL monitor systems. This combination of monitors covers the entire range of known AHL signal molecules (Andersen et al., 2001; Ravn et al., 2001). However, no responses in any

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**Fig. 1.** Schematic drawing of the genetic arrangements and mini-Tn5 insertions in the S. proteamaculans B5aN mutants. S. proteamaculans AC1 is a B5aN derivative containing a mini-Tn5 insertion in sprI; S. proteamaculans AC2 is an AC1 derivative containing mini-Tn5 insertions in sprI and lipB. (a) Orientation of the sprI and sprR genes and position of the kanamycin-resistance gene in S. proteamaculans AC1 and S. proteamaculans AC2. (b) Orientation of the sla4 and lipB genes and the position of the 'luxAB reporter gene insertion in S. proteamaculans AC2.
of these monitors were detected (data not shown). Furthermore, cloning of chromosomal DNA from the wild-type S. proteamaculans B5aN digested with three different restriction enzymes and subsequent TLC analysis on extracts from AHL-producing Escherichia coli clones did not indicate the presence of additional AHL-synthase-encoding genes (data not shown). Taken together, these results strongly suggest that sprl encodes the sole AHL-synthesizing enzyme in S. proteamaculans.

No lux-box-like regulatory element (inverted repeats) could be identified within the region spanning 400 bp upstream of the translational start codon of the sprl gene. Thus, since Ravn et al. (2001) were unable to detect autoinduction in the synthesis of 3-oxo-C6-HSL in S. proteamaculans B5a, we suggest that classical V. fischeri autoinduction of the sprl gene does not take place in this strain. However, it should be noted that lux box sequences are not a mandatory requirement for R-protein binding, e.g. CarR-directed activation of carA in Erwinia carotovora takes place in the absence of an obvious lux box sequence (Welch et al., 2000).

The sprl open reading frame (ORF) encodes a putative protein of 210 aa with a predicted molecular mass of 24.1 kDa (GenBank accession no. AAK76733). Sprl showed its highest degree of relative sequence similarity (79%) to EsrI of Pantoea stewartii (GenBank accession no. P54656). The degrees of relative sequence similarity to other LuxI homologous proteins were 77% to SpnI of S. marcescens (GenBank accession no. AAN52498), 61% to SwrI of S. liquefaciens MG1 (GenBank accession no. P52989) and 42% to LuxI of V. fischeri (GenBank accession no. CAA68562). Adjacent of the sprl gene, a reading frame showing similarity to the family of luxR homologous genes was located (Fig. 1). The two genes are transcribed convergently and their reading frames contain 23 overlapping bases. Interestingly, a similar genetic arrangement has been reported in various members of the Enterobacteriaceae, including Pantoea stewartii and S. marcescens (Beck von Bodman & Farrand, 1995; Horng et al., 2002). By contrast, the luxI and luxR genes of V. fischeri are transcribed divergently (Engbrecht & Silverman, 1984). The sprR ORF encodes a putative protein of 249 aa with a predicted molecular mass of 28.3 kDa (GenBank accession no. AAK76734), and it showed the highest degree of relative sequence similarity (86%) to SpnR of S. marcescens (GenBank accession no. AAN52499). The degrees of relative sequence similarity of SprR to other LuxR homologous proteins were 84% to EsrR of Pantoea stewartii (GenBank accession no. P54293), 66% to SwrR of S. liquefaciens MG1 (L. Eberl, M. K. Winson, C. Sternberg, G. S. Stewart, G. Christiansen, S. R. Chhabra, B. Bycroft, P. Williams, S. Molin & M. Givskov, unpublished data) and 45% to LuxR of V. fischeri (GenBank accession no. S06314). A helix-turn-helix motif could be identified in the C-terminal part of SprR by similarity to other R-proteins. Surprisingly, the putative I- and R-proteins of the two phylogenetically closely related Serratia species S. liquefaciens MG1 and S. proteamaculans B5aN exhibit an unexpectedly low degree of relative sequence similarity. Though speculative, the high degree of relative sequence similarity to SpnR and EsrR of S. marcescens and Pantoea stewartii, respectively, suggests that SprR might function as a repressor analogous to these R-proteins. The regulatory properties of SprR are currently under investigation.

The quorum sensing regulon

To obtain an estimate of the number of proteins that are responsive to 3-oxo-C6-HSL in S. proteamaculans B5aN, the protein patterns of S. proteamaculans AC1 (sprl mutant) grown in the absence and presence of 3-oxo-C6-HSL were compared by means of two-dimensional PAGE (Fig. 2). The cells were grown in minimal media and harvested in the early-stationary phase. Two-dimensional PAGE analysis of the intracellular proteins from the sprl mutant (AC1) demonstrated a complex 3-oxo-C6-HSL-directed protein expression pattern. Out of a total of approximately 400 separated proteins, a subset of 23 proteins was found to be repressed in the presence of 3-oxo-C6-HSL and induced in the absence of 3-oxo-C6-HSL (Fig. 2b, c). Yet another subset of 16 proteins was found to be repressed in the absence of 3-oxo-C6-HSL and induced in the presence of 100 nM of 3-oxo-C6-HSL (Fig. 2b, c). The wild-type (B5aN) displayed a protein expression profile that was similar to the protein expression profile of the sprl mutant grown in the presence of exogenously added 3-oxo-C6-HSL (Fig. 2a, c). This analysis, therefore, suggested the presence of a quorum sensing system in control of a minimum of 39 proteins. Quorum sensing thus governs control over approximately 10% of the detectable proteins. However, since the present analysis was performed on early-stationary-phase cells, it is an underestimate of the total number of proteins produced during the growth cycle and cannot represent the entire proteome. It has been estimated that 3–4% of the genes in Pseudomonas aeruginosa are under quorum sensing control (Whiteley et al., 1999). Global protein expression analysis of the quorum sensing regulon in S. liquefaciens MG1 demonstrated that at least 28 proteins are subjected to control by a C4-HSL-dependent regulatory quorum sensing system (Givskov et al., 1998). Thus the observation that a minimum of 39 proteins are controlled by a 3-oxo-C6-HSL-responsive quorum sensing system in S. proteamaculans B5aN is in good agreement with similar studies. The pleiotropic 3-oxo-C6-HSL-induced changes in the profile of intracellular proteins indicate that the putative R-protein might work as a repressor on one subset of target genes and yet as an activator on a second subset of the target genes. Alternatively, the putative SprR protein might work in conjunction with another regulatory component to modulate gene expression at the transcriptional level. Similar complex signal-molecule-induced expression profiles have been observed in Yersinia enterocolitica (Throup et al., 1995) and S. liquefaciens MG1 (Givskov et al., 1998). Multiple luxR homologous genes have been identified in S. marcescens, Erwinia carotovora and Pseudomonas
aeruginosa (Latifi et al., 1995; Salmond et al., 1995; Thomson et al., 2000). Thus, we cannot exclude the possibility that an additional luxR homologous gene is present in S. proteamaculans.

The lipB transporter is a quorum sensing target

A collection of S. proteamaculans AC1-derived secondary transposon mutants were generated by randomly inserting the promoterless luxAB-Sm cassette into the chromosome and subsequently screening for 3-oxo-C6-HSL-responsive mutants. One such mutant, S. proteamaculans AC2, had no detectable proteolytic activity on LB plates supplemented with skim milk powder. Southern blot analysis confirmed that only one copy of the luxAB cassette had been integrated into the chromosome (data not shown). DNA sequence analysis of the region upstream of the luxAB cassette located the insertion to 126 bases downstream from the putative translational initiation codon of a gene the partial gene product of which showed 93% identity and 99% similarity to the LipB proteins of S. marcescens (GenBank accession no. BAA08631) and S. liquefaciens MG1 (GenBank accession no. AAA02245). Upstream of the proposed lipB gene of S. proteamaculans, the gene product of a partially sequenced gene showed 91–92% similarity and 83–86% identity to the surface-layer proteins SlAA of S. marcescens and S. liquefaciens MG1, respectively (GenBank accession nos BAA35525 and AY007218). A schematic view of the genetic organization is shown in Fig. 1(b). Apart from S. marcescens (Kawai et al., 1998) and S. liquefaciens MG1 (Riedel et al., 2001), this genetic arrangement has also been reported for Caulobacter crescentus (Awram & Smit, 1998) and Campylobacter fetus (Thompson et al., 1998). Given the highly conserved gene organization, and the sequence and functional conservation between the Serratia spp., we suggest that lipB in S. proteamaculans is the first gene in a three-gene operon consisting of lipBCD. Although we cannot exclude the possibility that the observed phenotypic
Consequences of the insertion are caused by polar effects, the products of all three genes are known to be required for successful secretion of proteins (Binet et al., 1997).

Transcription of the lipB promoter was investigated throughout the growth cycle (LB medium) by measuring the specific activity of bioluminescence activity from the chromosomal lipB::luxAB fusion in the AC2 double mutant (Fig. 3). Induction of lipB transcription was observed at an OD450 value of approximately 3-5, which is in the late-exponential phase of growth. The addition of 100 nM of 3-oxo-C6-HSL resulted in a steeper induction profile where induction occurred at a significantly lower cell density (OD450 value of approximately 2-5), leading to an approximately 50% increase in specific activity (Fig. 3). The elevated, 3-oxo-C6-HSL-induced, transcription from the lipB promoter suggests that the lipB transporter is a target for the quorum sensing regulatory system in S. proteamaculans B5aN. By definition, the expression of quorum-sensing-regulated genes is affected when the cell population reaches a certain size. This reflects the requirement for signal molecule accumulation. However, exogenous addition of 3-oxo-C6-HSL to the sprl lipB double mutant (AC2) in the early-exponential phase did not lead to immediate induction of lipB, indicating that expression of lipB requires additional regulatory elements. The delayed mode of induction is in contrast to the direct induction of the luxI promoter from V. fischeri observed in LuxR-based AHL monitor systems (Andersen et al., 2001), but is in accordance with observations made with the quorum-sensing-controlled genes swrA and lipB in S. liquefaciens MG1 (Lindum et al., 1998; Riedel et al., 2001) and the induction of carA in S. marcescens (Thomson et al., 2000). Interestingly, both immediate and delayed responses to AHL signal molecules have been reported in Pseudomonas aeruginosa (Whiteley et al., 1999).

**Investigation of AHL-regulated exoenzymes and exoproteins**

The wild-type (B5aN), sprl mutant (AC1) and sprl lipB double mutant (AC2) were grown in LB medium until stationary phase (OD450 value of approximately 10) in the absence or presence of 3-oxo-C6-HSL. The filter-sterilized culture supernatants were subjected to protein analysis. The enzymatic activities were subjected to zymogram analyses. Zymograms are not quantitative; however, they do offer a rough estimate of the varieties and approximate molecular masses of active enzymes. The total enzymic activities of chitinase, lipase and protease were analysed with quantitative assays. In addition, the exoprotein profiles were investigated by means of SDS-PAGE.

The chitinase zymogram (Fig. 4a) presented one band with chitinolytic activity with an approximate molecular mass of 48 kDa. Production and secretion of several exoenzymes have been reported in S. marcescens, and at least three different chitinase-encoding genes have been identified: chiA encodes a 57 kDa enzyme, chiB encodes a 52 kDa enzyme and chiC encodes a 48 kDa enzyme (Watanabe et al., 1997). We therefore presume that the chitinase from S. proteamaculans corresponds to ChiC of S. marcescens. The presence of a 48 kDa chitinolytic band in spent media from the lipB mutant indicates that the chitinase is secreted via a LipB-independent transporting system and that the chitinase is not subjected to proteolytic processing by LipB-dependent proteases. Gal et al. (1998) have reported the proteolytic processing of a 52 kDa chitinase into an active 35 kDa enzyme by S. marcescens. The quantitative enzymic assays for chitinolytic activity (Fig. 4d) demonstrated that the chitinolytic activity from the sprl mutant (AC1) and sprl lipB double mutant (AC2) were similar, indicating that the chitinase is not secreted via the LipB transporter. Therefore, the observation that chitinolytic activities were twofold-inducible upon 3-oxo-C6-HSL addition suggested that the quorum sensing system controls transcription of the chitinase gene directly. We cannot, however, exclude the existence of a specific chitinase transporter which might be under the control of quorum sensing and thereby subjecting chitinase production to indirect quorum sensing control. The biological significance of chitinases includes biocontrol of growth and development of certain insects, nematodes and fungi (Herrera-Estrella & Chet, 1999). Serratia spp. are considered potential insect pathogens (Bucher, 1960); moreover, antifungal properties have been attributed to members of this genus (Kalbe et al., 1996). It is therefore likely that quorum sensing control of Serratia chitinases plays a key role in the expression of biocontrol phenotypes.

The lipase zymogram (Fig. 4b) presented one band with lipolytic activity, which had an approximate mass of 58 kDa, presumably corresponding to the 62 kDa lipase LipA from S. marcescens (Akatsuka et al., 1995). The quantitative enzymic assay for lipolytic activity (Fig. 4e) on spent culture media from the wild-type (B5aN) and the sprl mutant.

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**Fig. 3.** Liquid cultures of the sprl lipB double mutant, AC2, were grown in the absence (solid symbols) and presence (open symbols) of 100 nM of 3-oxo-C6-HSL. Growth (circles) was monitored at OD450, and the transcriptional activity of the lipB operon (triangles) was determined as specific activity (relative light units/OD450). Each data point represents the mean from three independent experiments; error bars represent the SEM.
(AC1) indicated that lipolytic activity was inducible by twofold upon addition of 3-oxo-C6-HSL; this is in contrast to the findings in *S. liquefaciens* MG1 (Riedel et al., 2001).

The protease zymogram (Fig. 4c) presented two bands with proteolytic activity with approximate masses of 52 and 48 kDa, respectively. Whether the two proteolytic bands represent two different enzymes or processed versions of the same one remains to be elucidated. The protease from *S. proteamaculans* B5aN has approximately the same mass as the 52 kDa metalloprotease PrtA from *S. liquefaciens* MG1 and *S. marcescens* (Nakahama et al., 1986; Riedel et al., 2001). The quantitative enzymic assay for proteolytic activity (Fig. 4f) on spent culture media from the wild-type (B5aN) and the sprl mutant (AC1) demonstrated a twofold induction upon AHL addition; this is in accordance with the observations made in *S. liquefaciens* MG1 (Riedel et al., 2001). The sprl lipB double mutant was devoid of proteolytic and lipolytic activity in our quantitative enzyme assays, indicating that both lipase and proteases are secreted through the lipB transporter. These findings support the observations made in *S. marcescens* and *S. liquefaciens* MG1 (Akatsuka et al., 1995; Riedel et al., 2001). Hence, since the activities of the lipB promoters and the proteases are inducible by AHLs in both *S. proteamaculans* B5aN and *S. liquefaciens* MG1 (Riedel et al., 2001), we propose that quorum sensing regulation of the lipB transporter is at least partially responsible for the observed induction of lipolytic and proteolytic activities.

SDS-PAGE analysis of the exoprotein profiles (Fig. 5) showed that seven exoproteins are present in spent culture media from the wild-type (B5aN) and the sprl mutant (AC1), but are undetectable in spent culture media from the sprl lipB double mutant (AC2), suggesting that the secretion of these proteins is dependent on the lipB transporter. We cannot exclude the possibility that the observed exoprotein profiles from the wild-type (B5aN) and sprl mutant (AC1) are a result of proteolytic degradation; likewise, the numerous exoprotein bands that are absent in the wild-type (B5aN) and sprl mutant (AC1) background, but present in the sprl lipB double mutant (AC2) background, could be caused by the lack of proteolytic activity by the lipB-dependent proteases. Therefore, the observed LipB-dependent exoproteins might represent fewer than seven distinct proteins.

**Fig. 4.** Analysis of extracellular enzymes produced by *S. proteamaculans* B5aN and different mutants visualized using zymograms (upper panel, a–c) and by measuring the specific enzymic activities (lower panel, d–f). (a) Chitinase zymogram; (b) lipase zymogram; (c) protease zymogram; (d) specific chitinolytic activity; (e) specific lipolytic activity; (f) specific proteolytic activity. Cultures were grown until stationary phase in LB medium in the presence or absence of 100 nM of 3-oxo-C6-HSL. Lanes: 1, Wild-type, B5aN; 2 and 3, sprl mutant, AC1; 4 and 5, sprl lipB double mutant, AC2. (d–f) Each bar of the specific enzymic activity analysis represents the mean from three independent experiments; error bars represent the SEM.
Quorum sensing regulation of food spoilage

The enzymic degradation of complex compounds is important in the deterioration of the quality of a range of foods (e.g., fruits, vegetables and raw milk), which may be caused by pectinolytic and proteolytic bacteria (Chatterjee et al., 1994; Tan & Miller, 1992). Other foods, such as fresh fish, spoil because of bacterial turnover of amino acids and other non-protein nitrogen compounds. In cold-smoked fish and vacuum-packed beef, a complex mixture of Gram-positive and Gram-negative bacteria develops (Borch et al., 1996; Paludan-Müller et al., 1998). The exact role and metabolism of the individual organisms in this mixture is not known. It is possible that the hydrolytic activity of the Gram-negative flora provides accessible nutrients for the non-hydrolytic Gram-positive flora dominated by lactic acid bacteria.

S. proteamaculans B5a was isolated from cold-smoked salmon, yet since the spoilage process of cold-smoked salmon is complex and involves interactions between lactic acid bacteria and Gram-negative bacteria (Jørgensen et al., 2000), we have investigated whether quorum sensing contributes to the spoilage process of food products where spoilage may be caused by the growth of psychrophilic members of the Enterobacteriaceae alone. We chose liquid milk in which Enterobacteriaceae spoilage can manifest itself as clotting due to proteolytic degradation of the casein micelles. Here, we present data demonstrating that quorum sensing regulation of protease and/or lipase contributes to the spoilage process of milk (Fig. 6). Samples of commercially purchased cows milk were inoculated with approximately $1 \times 10^6$ c.f.u. ml$^{-1}$ of the wild-type S. proteamaculans B5aN, the communication-deficient sprI mutant S. proteamaculans AC1 or the exolipase- and exoprotease-deficient sprI lipB double mutant S. proteamaculans AC2. Samples inoculated with S. proteamaculans AC1 or S. proteamaculans AC2 were grown in the absence and presence of 200 nM of 3-oxo-C6-HSL. After 18 h incubation at room temperature, samples inoculated with the wild-type and sprI mutant strain complemented with 200 nM of 3-oxo-C6-HSL were spoiled. However, samples inoculated with the sprI mutant in the absence of 3-oxo-C6-HSL, the sprI lipB double mutant inoculated without 3-oxo-C6-HSL and the sprI lipB double mutant in the presence of 200 nM of 3-oxo-C6-HSL appeared unspoiled. At the end point, all samples contained approximately $1 \times 10^7$ c.f.u. ml$^{-1}$, and all samples showed a pH value of 6.2. These results demonstrate that the quorum sensing system controls phenotypes involved in the spoilage of food. Moreover, since the sprI lipB double mutant could not be complemented by exogenous 3-oxo-C6-HSL, it is most likely that quorum sensing controls spoilage through transcriptional control of the secretion apparatus encoded.

Fig. 5. SDS-PAGE (12 %) analysis of extracellular proteins produced by the wild-type B5aN (lane 1), the sprI mutant AC1 (lanes 2 and 3) and the sprI lipB double mutant AC2 (lanes 4 and 5). Cultures were grown until stationary phase in LB medium in the presence or absence of 100 nM of 3-oxo-C6-HSL. Arrows indicate protein bands that were present in spent medium from B5aN and AC1, but not detectable in spent medium from AC2.

Fig. 6. Quorum sensing control of milk spoilage. Milk samples were inoculated with $1 \times 10^6$ c.f.u. ml$^{-1}$ of the S. proteamaculans wild-type or mutants. The samples were incubated at room temperature for 18 h. (A) Milk (negative control); (B) milk inoculated with the wild-type B5aN; (C) milk inoculated with the communication-deficient mutant AC1; (D) milk containing 200 nM of 3-oxo-C6-HSL inoculated with AC1; (E) milk inoculated with the communication-deficient and secretion-deficient double mutant AC2; (F) milk containing 200 nM of 3-oxo-C6-HSL inoculated with AC2.

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by lipB. We, therefore, suggest that spoilage is caused by the activity of these enzymes. Notably, proteolytic enzymes have been reported to cause clotting of milk (Payens, 1982). The fact that all samples contained the same concentration of bacteria emphasizes that the amount of spoilage metabolite (or spoilage-generating metabolites) produced per cell (or consortium of cells) is a critical parameter when evaluating the spoilage potential and activity of a strain.

In the present report, we have demonstrated that several hydrolytic enzymes produced by a typical member of a food spoilage flora are regulated by quorum sensing. Furthermore, we have demonstrated that quorum sensing is involved in the production of spoilage characteristics in situ on food products. Our results indicate that novel preservation techniques employing specific quorum sensing inhibitors may be developed (Givskov et al., 1996).

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